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ANNOUNCEMENT.

CORRECTION.

The accompanying Equation 22 is to replace Equation 22 on page 543, Vol. LII, No. 2, June, 1922, line 26. The slip is arranged to be pasted over the place occupied by the equation at present.

$$(22) \quad K'_a = [H^+] \frac{C - 0.8686\beta_M \pm \sqrt{(0.8686\,\beta_M - C)^2 - 0.7566\beta_M^2}}{0.8686\beta_M}$$

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deteriorated too far physically.   In practice we have allowed the
animals to remain on the deficient diet until their appearance
and loss of weight showed clearly that they were repeating the
usual behavior of animals on diets free from water-soluble B.
We then modified the diet by replacing a part of the dextrin by
the food substance which we desired to study.

Test of Onion Root for Water-Soluble B.

Two young rats were prepared as above described for testing
the value of onion roots for water-soluble B.   On the 29th day
the diet was modified by the introduction of 4.4 per cent of dry
onion roots.   One animal died almost as soon as this change was
made.   The other responded in a manner which is illustrated by
the curve in the accompanying chart (Lot 2973 D).   It is evident
that onion roots contain a small amount of water-soluble B, but
not more than a similar amount of a cereal grain.   It would re-
quire about 15 to 20 per cent of whole wheat to cause the recovery
and resumption of growth in a rat in the condition of the one
described.   About 3 per cent of wheat germ would suffice to bring
about this result.

A Test of a Mushroom (Agaricus campestris) for Water-Soluble B.

Two young rats were prepared in the usual way by restricting
them to the diet which was satisfactory in all respects except
that it lacked water-soluble B.   At the end of the 4th week both
were definitely declining.   On the 29th day, 9 per cent of dried
Agaricus campestris was introduced into the food formula in place
of an equivalent amount of dextrin.   No other change was made
in the feed or management of the animals.   Their response with
increased physical strength and improved appearance was immed-
iate.   They began at once to grow, and continued to do so to
the end of the test, which lasted until one had increased in weight
from 85 to 148 gm.   The other increased from a body weight of
70 to 115 gm.   The mushroom feeding period extended over 5
weeks.   This indicates that the mushroom is an excellent source
of water-soluble B.   Coward and Drummond (5) have recently
reported that *Agaricus campestris* is almost devoid of fat-soluble A.

Ration of all groups in Period 1.

Casein	18.0
Salts (185)	5.7
Agar-agar	2.0
Dextrin	71.3
Butter fat	5.0

Period 2.

Lot 3265 O.
9 per cent of dry mushrooms replaced part of dextrin

Lot 3265 O

Period 2.

Lot 3364 A.
8 per cent of dry Indian-pipe replaced part of dextrin

Lot 3364 A

Period 2.

Lot 3304 A.
15 per cent of dodder replaced part of dextrin

Lot 3304 A

Period 2.

Lot 2973 D.
4.4 per cent of onion sprouts replaced part of dextrin

Lot 2973 D

GRAMS
160
120
80
40

Weeks

CHART 1.

Test of Indian-Pipe (Monotropa uniflora) for Water-Soluble B.

Our supply of material was limited to 20 gm. of the dry substance of this plant. We, therefore, limited our test to a single animal. This was restricted as above described to the experimental diet to bring it into a condition where its body was depleted of water-soluble B. On the 29th day, when it was declining in vigor in the usual manner, the diet was modified so as to include 8 per cent of *Monotropa uniflora* in place of an equivalent amount of dextrin. There was no response with growth, but the condition of the animal improved, and it remained active over a period of 7 weeks. At this point the experimental ration was exhausted and the test was discontinued. The experiment is inconclusive, but indicates that *Monotropa uniflora* probably contains a moderate amount of water-soluble B for the rat's life was prolonged beyond the average of those which we have seen restricted to this diet without a source of water-soluble B.

Test of a Non-Chlorophyll-Producing Parasitic Plant (Cuscuta gronovii) for Water-Soluble B.

Gronovius' dodder (*Cuscuta gronovii*), is a parasitic plant which is devoid of chlorophyll, and can be secured in large amounts with little difficulty. It was, therefore, thought worth while to test it for its content of water-soluble B. To this end we restricted six young rats to the deficient diet until they were in a state of decline owing to specific starvation for water-soluble B. About the 35th day 15 per cent of dry dodder was introduced into the diet in place of an equivalent amount of dextrin. The animals all died within a week after this change was made, apparently because of toxicity of the dodder.

CONCLUSIONS.

We have tested the onion root, a structure which contains no chloroplasts, for the presence of water-soluble B, and have found it to contain a certain amount of this dietary essential. This warrants, we believe, the conclusion that the substance, water-soluble B, is not concerned with the structure of the chloroplast.

The mushroom, *Agaricus campestris*, proved to be a good source of water-soluble B.

Indian-pipe, *Monotropa uniflora*, a non-chlorophyll-bearing plant, gave results which were inconclusive when tested by our method for the presence of water-soluble B.

Dodder, *Cuscuta gronovii*, proved toxic, and caused the death of the experimental animals. It cannot be determined from our experiments whether this parasitic plant does or does not contain water-soluble B.

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5. Coward, K. H., and Drummond, J. C., The formation of vitamin A in living plant tissues, *Biochem. J.*, 1921, xv, 530.

GLACIAL ACETIC ACID AS A SOLVENT FOR THE ANTI-NEURITIC SUBSTANCE, WATER-SOLUBLE B.*

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Up to the present time water has proved the only effective solvent for the antineuritic substance, water-soluble B. It is usually stated that this substance is also extracted from natural foods by hot alcohol, but this is, strictly speaking, not true, for absolute alcohol does not extract a sufficient amount of the substance from such vitamin-rich substances as wheat germ to give satisfactory results in growth experiments on young rats. Alcohol containing water is a solvent for the vitamin, and indeed, the higher the content of water, the better the solvent action of the alcohol.

Benzene, alcohol, ether, ethyl acetate, and acetone have been tested thoroughly as solvents for water-soluble B, employing the rats as the test organism (1). Our later studies have shown us that while it is possible to extract from the alcoholic extract of natural foods by means of hot benzene a sufficient amount of water-soluble B to give a positive test for the vitamin, this solvent is too poor to have any practical value as an aid to the isolation of the substance.

McCollum and Simmonds (1) have discussed the relative merits of the rat and the pigeon as subjects for testing the potency of any preparation supposed to contain this substance. It is their view that the only satisfactory method of conducting this test

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* Since this paper was sent to the publishers we have noted in the Vitamine Manual by W. H. Eddy that he has used glacial acetic acid as a solvent for water-soluble B.

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is to restrict a young rat for a period to a diet which is satisfactory in all respects except for the lack of water-soluble B, and to pass judgment as to the presence or absence of the vitamin in question in a given preparation by the response or failure of response with growth after the animal has declined to a point near collapse. If a rat, in an enfeebled condition, under such conditions returns on the administration of a preparation of water-soluble B to a state of vigor, resumes growth at normal rate, and exhibits a normal appearance, there can be no doubt that the vitamin in question has been supplied. On the other hand, there is much evidence that the traditional test for this vitamin, using the pigeon as a subject, is unreliable, and may lead to serious errors in judgment concerning the properties of various preparations which may be made for the study of this most interesting substance. Dutcher (2) has pointed out that physiological stimulants of several kinds, which McCollum and Koch (3) have shown to possess no power to replace the substance water-soluble B, may induce temporary improvement in a pigeon suffering from acute polyneuritis. McCollum and Simmonds (4) have convinced themselves that several of the substances which have been reported to possess antineuritic properties when tested on pigeons, have no value as a source of water-soluble B when tested under the conditions described above, using the rat as a subject, *and involving the element of growth as an essential feature of the test.*

We were fortunate in discovering, about 2 years ago, that glacial acetic acid is an excellent solvent for water-soluble B, and by the aid of this solvent have made many preparations which are very potent in the dietary essential in question. We are employing glacial acetic acid in connection with other solvents which do not dissolve the vitamin in connection with our efforts to isolate this substance.

For many obvious reasons the use of glacial acetic acid is a fortunate one. It is cheap and readily obtainable. Its use results in a preparation which is solid and easily powdered, whereas water or alcohol yields a viscid, gelatinous, semisolid mixture containing the antineuritic factor. Acetic acid is a water-miscible solvent and easily penetrates biological material, animal or plant, wet or dry, in the form of small lumps or in powder. It has a comparatively low boiling point, 118°C., and hence

can be conveniently distilled off under slightly reduced pressure
at a temperature at or below the boiling point of water without
inactivating the vitamin. Since the solvent is an acid it may
aid in inhibiting or preventing the oxidation of water-soluble B,
which may be facilitated by stirring or exposure to air, especially
at high temperatures. Many organic compounds, monosaccha-
rides, disaccharides, phenols, etc., easily undergo oxidation in an
alkaline medium but this process suffers retardation in the pres-
ence of acid. A solution of water-soluble B in glacial acetic
acid should be by *a priori* reasoning very active even after long
standing. This we have proved to be the case. A very unique
property of glacial acetic acid from the standpoint of the isola-
tion of vitamin in the pure state lies in its ability to precipitate
carbohydrates. Still another and not unimportant considera-
tion arguing for the use of glacial acetic acid is its miscibility in
all proportions with ether—a condition which enhances the value
of the latter as a means of purifying the antineuritic factor by
removal by precipitation of a large amount of inactive material
from the active mixture.

The following brief statement of a procedure which will be
of interest to investigators in this field for separating water-soluble
B from a large part of the components of a natural food, is made
at this time, because if affords a new aid to inquiry in this direc-
tion. A typical test of the biological value of the preparation is
also included.

1,000 gm. of ground raw navy beans and 3,000 cc. of glacial
acetic acid were heated for 3 hours on the water bath. The mix-
ture was filtered hot on two layers of cloth, the residue washed
again with small quantities of 1,000 cc. of glacial acetic acid, and
heated with 3,000 cc. of glacial acetic acid for another 3 hour
period. The hot mixture was filtered on two layers of cloth and
washed with 1,000 cc. of glacial acetic acid, using small quantities
at a time. The filtrate was now passed through fluted paper. The
resulting, clear, reddish brown liquid was distilled at 70°C. under
reduced pressure and the residue dried with the electric fan.
The final result is a solid, which readily lends itself to powdering.

27.5 gm. of solid extract prepared as described above, and cor-
responding to 250 gm. of navy beans, were dissolved with the
aid of heat in 1,000 cc. of glacial acetic acid. This was precipi-

tated with 5 volumes of ether. The precipitate, a solid, choco-
late-brown substance, was removed by filtration. The filtrate
was evaporated to dryness with a blast of air from an electric
fan, and left a chocolate-colored solid residue. The precipitate
(Preparation I) weighed 15.5 gm., and the residue (Preparation
II) from the evaporation of the filtrate weighed 12.0 gm. Each
of these preparations was tested for water-soluble B, using young
rats as test animals.

A group of young rats weighing about 45 to 70 gm. was restricted
to the following diet, which was complete except for the absence
of water-soluble B.

<div style="text-align:right">per cent</div>

Casein 18.0
Salt mixture (185) 3.7
Agar-agar 2.0
Dextrin 71.3
Butter fat 5.0

The casein employed had been carefully freed from almost
all traces of inorganic salts and vitamins by a method previously
described. It consists of washing the finely ground casein in
distilled water acidified to the extent of about 0.2 per cent with
acetic acid. During the 1st day four changes of tap water are
made. It is then soaked over night in acidified, distilled water.
The casein is daily removed on cheese-cloth and freed from most
of the liquid, then returned to fresh distilled water and acidified
again. This treatment is continued through 7 days. The salts
of the crude casein are thus caused to dialyze out of the granules,
and the impurities are completely washed from the swollen gran-
ules. Such a procedure we have found through extensive ex-
perience to result in a preparation which is free from demonstrable
amounts of any vitamin.

On the diet described small rats are able to increase in weight
in some cases for about 2 or 3 weeks. Their growth is then sus-
pended and they gradually become attenuated of form and en-
feebled. Many reach a stage where they toss the head backward
repeatedly, and at intervals show the signs of opisthotonos.
Finally, they may develop the symptoms of acute polyneuritis,
but some die without reaching this condition. When it is evident
that steady physical decline has set in, the animals are ready for

the administration of any curative preparation which it is desired to test, for they never improve if confined to the experimental diet as their sole source of nutriment.

Rats fed the experimental diet for 6 weeks, and which had begun to decline, were given Preparation I, equivalent to 50 per cent of beans in the diet. This preparation is the precipitate formed by pouring ether into a glacial acetic acid solution containing the glacial acetic acid-soluble matter derived from raw navy beans. The test was negative. The animals continued to decline and died. This indicates the nearly complete absence of water-soluble B from the precipitate in question.

Rats fed the experimental diet as above described were given, after decline had set in, the material which remained in solution when a filtered acetic acid extract of raw navy beans was poured into five times its volume of ether (Preparation II). The amount administered was equivalent to 50 per cent of beans in the diet. The animals responded in a manner fully as remarkable as they would have done had a liberal amount of a natural food containing water-soluble B been made to replace half of their experimental diet.

We do not desire at this time to discuss in detail the degree of potency of this and similar preparations, since we are carrying on further studies in the direction of perfecting a method of freeing as far as possible the vitamin, water-soluble B, from contaminating substances.

CONCLUSIONS.

Glacial acetic acid is the best organic solvent which we have yet found for the extraction of water-soluble B from plant materials. The active glacial acetic acid extract can be further concentrated by the removal by precipitation with ether of a large quantity of inactive material.

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A MODIFICATION OF THE BELL-DOISY PHOSPHATE METHOD.

By A. P. BRIGGS.

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The colorimetric phosphate method of Bell and Doisy,[1] if followed carefully as described, gives results which check gravimetric determinations, but this method has one objection; namely, that the alkaline blue color which is used for comparison in the colorimeter fades rather rapidly. For this reason it is not advisable to read more than about two determinations against the same standard. In the first stage of color production a stable green is produced in acid solution which is proportional to the phosphorus present. This color was not used by Bell and Doisy[1] for comparison (personal communication from Dr. Doisy) because with either urines or trichloroacetic acid blood filtrates there is an occasional turbidity produced when the acid molybdate solution is added which interferes with the color comparison, due to a precipitate of undetermined nature. In attempting to overcome these difficulties, it was found by the writer that by a small modification during the trichloroacetic acid precipitation of blood or plasma, the turbidity can be avoided, thus allowing the use of the acid solutions for color comparison. When blood or plasma is diluted with 3 volumes of water and 1 volume of 20 per cent trichloroacetic acid in an Erlenmeyer flask, shaken vigorously for a few seconds, and then allowed to stand for about 10 minutes before filtering, the filtrates give with acid molybdate and hydroquinone perfectly clear green colors. The supposition is that when the blood is diluted in a volumetric flask and mixed merely by inverting a few times, a small amount of protein gets through into the filtrates and this forms a precipitate with

[1] Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, 1920, xliv, 55.

the molybdic acid. A parallel series of determinations on plasma filtrates showed that identical results are obtained, whether the acid green color is used for comparison or the comparisons made of the alkaline blue according to the technique of Bell and Doisy. The green color is considerably less intense, and accurate comparison is with low phosphorus plasmas difficult; but the stability of color offsets this disadvantage.

It has also been recently observed that when a little sodium sulfite is added to an acid solution containing phosphate and molybdate that the subsequent addition of hydroquinone causes the formation of a blue instead of a green color and of an intensity considerably greater than the green. This color does not depend upon reduction of the molybdic acid by SO_2 since sodium sulfite, hydroquinone, and acid molybdate solutions when mixed give no color. The use of these modifications gives a clear blue, non-fading color for comparison, the proportionality of which is exact over a wide range. The intensity of the color allows the determination of phosphates in 1 cc. of plasma. The following technique is that used for blood or plasma: A measured volume of plasma is transferred to a small Erlenmeyer flask, diluted with 3 volumes of water and 1 volume of 20 per cent trichloroacetic acid. The flask is stoppered with the thumb, shaken vigorously for a few seconds, and after standing about 10 minutes, the contents are transferred to a dry ashless filter. The filter funnels rest in long Pyrex test-tubes and are covered by watch-glasses to prevent loss by evaporation. For the determination, transfer 5 cc. of the filtrate, equivalent to 1 cc. of plasma, to a 10 cc. volumetric flask or a long test-tube graduated at 15 cc. For the standard, transfer 2 cc. of the diluted phosphate solution, to a similar flask or tube. To each then add 2 cc. of the molybdate solution, 1 cc. of the sodium sulfite solution, and 1 cc. of the hydroquinone solution, and dilute with water to the mark. Allow them to stand about 30 minutes for color production and compare in the colorimeter.[2]

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[2] It is not necessary to add trichloroacetic acid to the standard to balance that of the filtrate. It is necessary, however, to have the acidity within certain limits for color production. Sufficient acid is provided by 2 cc. of the molybdate reagent for the formation of ammonium phosphomolybdate and its subsequent reduction; on the other hand if the total acidity after addition of all reagents is more than about 2 N then no color will be obtained.

Solutions Used.

Standard Phosphate Solution for Urine.—This solution contains 0.4394 gm. of dry KH_2PO_4 per liter. 1 cc. is equivalent to 0.1 mg. of phosphorus. Chloroform is added as preservative.

Standard Phosphate Solution for Blood.—25 cc. of urine phosphorus standard is diluted to 200 cc. and preserved with chloroform. 2 cc. of this solution are equivalent to 0.025 mg. of phosphorus.

Molybdate Solution.—25 gm. of ammonium molybdate are dissolved in 300 cc. of water. To this are added 200 cc. of water containing 75 cc. of concentrated H_2SO_4.

Hydroquinone Solution.—0.5 gm. of hydroquinone is dissolved in 100 cc. of water and a drop of concentrated H_2SO_4 added to retard oxidation. 1 cc. of this solution provides an abundant excess even in the determinations on urines high in phosphorus.

Sulfite Solution.—This solution contains 20 per cent sodium sulfite. It should be kept well stoppered or made fresh.

The following test was carried out to test the proportionality between the phosphorus present and the color produced by the new technique. Amounts of the standard phosphate solution varying from 7 to 25 cc. were transferred to 100 cc. volumetric flasks. To each were added in succession 5 cc. of the molybdate solution, 1 cc. of the sulfite solution, and 1 cc. of the hydroquinone solution. They were then diluted with water up to the mark, mixed by inverting a few times, and allowed to stand about an hour for color production. Each was then compared with the one containing 15 cc. The average of several readings of each solution showed a perfect proportionality over this range.

The modified technique was compared with the Bell-Doisy procedure on ten rabbit bloods with the results given in Table I.

The whole rabbit blood was taken because of the inconsistencies recently reported by Myers and Shevky[3] with the Bell-Doisy technique on "many" rabbit bloods. All of these filtrates gave perfectly clear blue colors by the modified technique and clear bluish green colors after the addition of molybdate and hydroquinone by the Bell-Doisy technique.

The modified technique is also applicable to urines. Comparison with the Bell-Doisy technique was made on a few urines

[3] Myers, B. A., and Shevky, M. C., *J. Lab. and Clin. Med.*, 1921-22, vii, 176.

according to the following procedure: Take 1 to 5 cc. of acidified urine or an amount equivalent to about 0.5 mg. of P, in a 100 cc. volumetric flask. In a similar flask, take 5 cc. of the urine P standard. Dilute each with water up to about 80 cc. Then add to each 5 cc. of the molybdate solution, 1 cc. of the sulfite solution,

TABLE I.

Comparison of the Bell–Doisy Method with Modified Technique on Ten Rabbit Bloods.

Blood.	Bell-Doisy method.	Author's modification.
	mg. per 100 cc.	*mg. per 100 cc.*
1	8.19	8.57
2	5.93	6.22
3	9.90	9.88
4	10.20	10.01
5	8.19	8.26
6	8.76	8.57
7	7.50	7.50
8	10.05	10.07
9	8.51	8.66
10	8.51	8.63

TABLE II.

Analysis of Three Urines by the Two Methods.

Urine.	Bell-Doisy method.	Author's modification.
	gm. per liter	*gm. per liter*
1	1.21	1.14
2	1.28	1.32
3*	1.54	1.56

* Urine 3 gave a precipitate of undetermined nature which was removed by centrifugation while the color was developing.

and 1 cc. of the hydroquinone solution. Dilute each with water up to the mark and allow to stand about ½ hour for color production. The results are given in Table II.

Addendum.—After this paper had been sent to press, it was shown by Denis[4] that oxalates and citrates interfered with the formation of the blue color of the Bell-Doisy method. It has been found here, however, that such amounts of citrates or oxalates as are used to prevent clotting of blood do not interfere with the formation of the acid blue and so no error is introduced in the determination by the modified technique.

[4] Denis, W., and von Meysenbug, L., *J. Biol. Chem.*, 1922, lii, 1.

A COLORIMETRIC DETERMINATION OF BLOOD CHLORIDES.

By M. L. ISAACS.

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The following colorimetric method makes use of the Folin and Wu filtrate, is rapid, and employs a permanent standard.

Principle.

Since silver chloride is about one-thirtieth as soluble as red silver chromate, it follows that silver chromate will dissolve in a solution of chlorides; the silver being reprecipitated as chloride, while a certain amount of yellow chromate goes into solution according to the following equation:

$$2NaCl + Ag_2CrO_4 \rightarrow 2AgCl + Na_2CrO_4$$
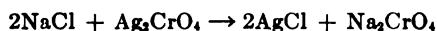
The reaction is familiar to the analytical chemist in the Mohr titration of chlorides. Silver chromate imparts only a very slight color to distilled water.

Reagents.

1. Silver chromate. (Red modification.) This is best prepared by adding slowly 200 cc. of a 5.5 per cent solution of potassium chromate to 100 cc. of a boiling solution of silver nitrate (10 per cent). The silver chromate settles out rapidly. Drops of the chromate solution are added until there is a slight excess of chromate, which gives the solution a yellow color. After cooling, the silver chromate is thoroughly washed with distilled water and finally air-dried on a Buchner funnel.

2. Magnesium carbonate.

3. Ammonium hydroxide, 2 per cent.

Procedure.

10 cc. of the Folin and Wu filtrate are pipetted into a small conical centrifuge tube (which has been previously cleaned with

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warm chromic acid solution). A pinch of magnesium carbonate is added to insure neutrality of the liquid. The contents of the tube are stirred with a thin glass rod. A small quantity (about 0.05 gm.) of silver chromate is introduced and thoroughly stirred into the solution. If all the red particles disappear more chromate must be added. After washing off the stirring rod into the tube, the tube is centrifuged for 2 minutes. The contents are then decanted through a small filter, into a 25 cc. volumetric flask, great care being taken not to disturb the residue at the bottom of the tube. After the addition of 10 cc. of water to the tube, the centrifuging is repeated for 5 minutes. The contents of the tube are then filtered into the volumetric flask. The solution has a slight turbidity which is cleared up by the addition of 1 cc. of a 2 per cent ammonium hydroxide solution. Enough water is added to bring the solution to the mark. After mixing, comparison is made with a standard potassium chromate solution containing 0.4 gm. of the salt per liter. The value of this standard may be found by employing 5 cc. of a 0.02 N solution of sodium chloride in place of 10 cc. of blood filtrate.

Since yellows are difficult to match, the colors can be viewed through a blue glass, as suggested by Michaelis.[1]

With the chromate solution used above, the chromate being 99.4 per cent pure, with the colorimeter standard at 20, the following formula applies:

$$\frac{11.730}{\text{Unknown reading}} = \text{mg. sodium chloride per 100 cc. blood}$$

In the following blood filtrates the method of Whitehorn[2] was used as a rapid check.

Sample.	Whitehorn's method.	Colorimetric method.
1	484	484
2	490	502
3	517	516
	517	520
4	492	504
5	477	489
6	503	504

[1] Michaelis, L., Deutsch. med. Woch., 1921, xlvii, 465.
[2] Whitehorn, J. C., J. Biol. Chem., 1920–21, xlv, 449.

Using 5 cc. of filtrate good results were obtained, but the colors were hard to match.

Mention should be made of the possible effects of other salts in the filtrate. Silver phosphate is slightly less soluble than silver chromate and it would be expected that silver chromate would dissolve in a solution containing phosphate. This does take place, but in very dilute solutions such as the blood filtrate the color develops very slowly. Furthermore, the phosphates of the blood are probably acid phosphates which do not react with the chromate. This was shown by adding 0.0010 gm. of monosodium hydrogen phosphate to 5 cc. of blood filtrate, an amount which would correspond to an extreme case of phosphate retention. As a check, 5 cc. of the same filtrate were taken and both filtrates were treated with magnesium carbonate and silver chromate. No difference of color could be observed. Other silver salts which are less soluble than the chromate are either absent or present in negligible quantities in the filtrate.

Using the principle of differential solubilities it is hoped that methods can be worked out for calcium, potassium, magnesium, phosphates, and other ions.

DIETARY FACTORS INFLUENCING CALCIUM ASSIMILATION.

II. THE COMPARATIVE EFFICIENCY OF DRY AND GREEN ALFALFA IN MAINTAINING CALCIUM AND PHOSPHORUS EQUILIBRIUM IN MILKING COWS.*

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(*From the Department of Animal Husbandry, University of Wisconsin, Madison.*)

The fact that green plant tissue (1) contains a more liberal quantity of a vitamine favoring calcium assimilation than does dried plant tissue led us to a study of this problem in its relation to the calcium and phosphorus metabolism of milking cows. We had in earlier work (2) observed very marked negative calcium balances in a liberally milking cow receiving a dry cereal straw as her roughage. In this case the daily calcium oxide intake was 25 gm. with a daily milk yield of 30 to 38 pounds.

In extensive experiments involving a number of years of work, Forbes (3) and his associates have observed negative calcium balances with milking cows receiving the best of dried forage in respect to its calcium content; namely, alfalfa or clover hay. In some of these cases the daily calcium oxide intake was as high as 175 gm. with a daily milk production of 51 pounds and a utilization of but 40 gm. of calcium oxide for this milk production; yet a negative calcium balance was reported.

Meigs, Blatherwick, and Cary (4) have presented data indicating that a dry, pregnant cow is probably not assimilating sufficient

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

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calcium from a calcium-rich ration such as alfalfa hay, corn silage, and a grain mixture for a positive calcium balance, but is actually transferring calcium salts from her skeleton for fetal skeleton-building.   Meigs and his associates are inclined to interpret the observed negative calcium balances as only temporary and merely induced by the nervous disturbances of the animal incidental to the collection of the excreta and general interference with the freedom of the animal.

Of the above observations those of Forbes were outstanding and his work made it appear probable that a liberally milking cow fed the richest available carrier of calcium as forage, such as dry alfalfa hay, would nevertheless be compelled to draw on her own mineral reserves for maintenance of milk production.   We accepted this point of view and formulated the hypothesis that the negative calcium balance of a milking cow receiving dry alfalfa hay would be turned to a positive one if the alfalfa were fed *fresh* and *green*.   In fact, it appeared probable to us that it must be during the period of green pasturage that the depleted mineral reserves of dairy cattle, incident to a long winter milking period, would be replenished.   True, the factor of the nature of the green grasses would be important because it would be entirely possible that if the grasses were of those varieties naturally low in lime and in addition had grown on an acid soil, the factor of mere greenness and consequently richness in a vitamine assisting calcium assimilation would not be sufficient to establish positive calcium balances with liberally milking animals.   Further, it appeared to us unbelievable that the dairy cattle of our country were in constant negative calcium balance, or else a low average milk production and an early termination of the life of the individual as a milk producer must follow, an assumption that does not appear to be supported by facts.   However, it is very probable that a condition of negative calcium balance in milking cows does widely prevail especially in the winter time and in regions where low calcium-carrying roughages are used—a condition that must have an important bearing on the yield of milk, resistance to disease, and reproduction (5).

Our plan was to feed liberally milking cows dried alfalfa hay plus silage and a grain mixture over a period of time sufficient in length to establish the assumed negative calcium balance and

then to replace the dried alfalfa hay with fresh, green alfalfa in
an amount equivalent in dry matter to the dry hay.   The results
we secured in our first experiments with *dry* alfalfa hay support
the view that it is possible to maintain calcium equilibrium in
high milking cows with such a roughage.   These particular results
are contrary to the findings of Forbes and his associates as well
as contrary to results we, ourselves, have secured in later experi-
ments with another alfalfa hay (data to be published later).
Equilibrium or positive calcium balances were obtained with this
dry alfalfa hay as well as with the *green* and succulent alfalfa.

EXPERIMENTAL.

For this work three pure-bred Holstein cows were used.   No. 1
weighed 1,372 to 1,468 pounds; No. 2, 1,093 to 1,107 pounds;
and No. 3, 1,284 to 1,293 pounds, at the beginning and end of the
experiment which ran from May 11th to July 5th, 1921.   No. 1
had freshened in December, 1920; No. 2 in October, 1920; and No.
3 in May, 1920.

The animals were confined to metabolism stalls with quantita-
tive collection of the excreta and milk.   The collection of the
excreta, as in all of our experiments of this character, was always
done by men working in 8 hour shifts.

No. 1 was with calf and due to freshen September, 1921.   She
had been on a poor mixed hay (low in calcium) before being placed
in the experiment and it is apparent from the data in Table II
that she was in condition for a more pronounced storage of calcium
and phosphorus than either of the other two animals.   Nos. 2 and
3 had been receiving dry alfalfa hay for 20 weeks prior to being
taken into the experiment and did not show as large a storage as
did No. 1. Cows 2 and 3 were not with calf.

Calcium determinations were made on all the feeds, milk, and
excreta by the McCrudden method.   Phosphorus was determined
in the feeds, milk, and feces after ashing in the presence of mag-
nesium nitrate.   In the urine phosphorus was determined by the
Neumann method, that is, after oxidation with nitric acid in the
presence of sulfuric acid.

The feeds used in the first period of 4 weeks duration were corn
silage, *dry* alfalfa hay, and a grain mixture made up of 60 parts of
yellow corn, 25 parts of wheat bran, and 15 parts of oil meal.
The alfalfa hay was of good quality, second cutting, *cured under*

caps, and grown on a southern Wisconsin farm. It retained a fairly bright green color and was judged as first quality. The alfalfa hay was chopped before feeding. For 3½ pounds of milk production 1 pound of the grain mixture was allowed. The daily ration consisted of 30 pounds of corn silage, 10 pounds of dry alfalfa hay, and an amount of the grain mixture proportional to the milk produced. On this basis No. 1 received 7 pounds of grain mixture daily, No. 2 received 13 pounds daily, and No. 3 received 9 pounds daily during the entire 8 weeks of the experiment and including both the dry and green alfalfa feeding periods. It should be noted that the daily allowance of silage and alfalfa hay was constant for the three animals during the entire time of the experiment.

TABLE I.

Calcium Oxide and Phosphorus Pentoxide Content of Feeds Used.

Material.	CaO	P₂O₅	Remarks.
	per cent	*per cent*	
Alfalfa hay........	1.82	0.73	Used in first period.
Corn meal........	0.027	0.59	
Wheat bran.......	0.170	2.68	
Oil meal..........	0.557	2.31	
Corn silage........	0.50	0.57	Air-dried condition.
Green alfalfa......	1.42–2.05	0.57–0.73	Analysis on air-dried material.

Following the 4 weeks of *dry* alfalfa hay feeding was a period of 4 weeks during which *fresh, green* alfalfa displaced the dry hay. The rest of the ration remained the same. The green alfalfa was cut daily from a field that had already been cut once and was for the most part in prime condition.

Water determinations were made daily on the green material in order to keep the dry matter of the ration derived from the green alfalfa as constant as possible and equivalent to the 10 pounds of air-dried hay fed daily in the first period. Further, samples of the green alfalfa were taken daily for calcium and phosphorus determinations. All the animals received distilled water. Common salt was fed *ad libitum*.

In Table I the calcium and phosphorus content of the feeds used are given. In the case of the corn silage, the percentages are as found on the air-dried material. No constant figures can

be given for the green alfalfa hay as it varied slightly from day to day; the CaO content of the air-dried material derived from the

TABLE II.

Record of Calcium Balance of Animal 1.

Period.	CaO in feces.	CaO in urine.	CaO in milk.	Total CaO excreted.	Total CaO intake.	Balance per week.	Balance per day.	Milk per week.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*lb.*

Dry hay period.

Period.	CaO in feces.	CaO in urine.	CaO in milk.	Total CaO excreted.	Total CaO intake.	Balance per week.	Balance per day.	Milk per week.
May 11–17......	451.54	1.76	178.20	631.50	784.94	+153.40	+21.91	160
" 18–24......	524.81	1.21	172.07	698.09	784.94	+ 86.85	+12.41	151
" 25–31......	534.32	1.32	156.06	691.70	773.23	+ 81.53	+11.36	137
June 1–7.......	537.08	0.94	136.37	674.39	773.23	+ 98.84	+14.12	122

Green hay period.

Period.	CaO in feces.	CaO in urine.	CaO in milk.	Total CaO excreted.	Total CaO intake.	Balance per week.	Balance per day.	Milk per week.
June 8–14......	563.97	1.09	110.23	675.29	768.81	+ 93.52	+13.36	98
" 15–21......	523.58	2.81	75.06	601.45	802.46	+201.01	+28.71	66
" 22–28......	613.42	2.22	65.28	680.92	1,025.37	+344.45	+49.21	64
" 29–July 5..	441.57	1.55	60.66	503.78	808.12	+304.34	+43.48	63

Record of Phosphorus Balance of Animal 1.

Period.	P_2O_5 in feces.	P_2O_5 in urine.	P_2O_5 in milk.	Total P_2O_5 excreted.	Total P_2O_5 intake.	Balance per week.	Balance per day.

Dry hay period.

Period.	P_2O_5 in feces.	P_2O_5 in urine.	P_2O_5 in milk.	Total P_2O_5 excreted.	Total P_2O_5 intake.	Balance per week.	Balance per day.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
May 11–17........	409.42	15.00	182.41	606.83	747.55	+140.72	+20.10
" 18–24........	397.15	17.35	170.00	584.50	747.55	+163.05	+23.29
" 25–31........	436.28	8.62	153.58	598.48	734.20	+135.72	+19.39
June 1–7........	527.18	11.36	134.70	673.27	734.20	+ 60.96	+ 8.71

Green hay period.

Period.	P_2O_5 in feces.	P_2O_5 in urine.	P_2O_5 in milk.	Total P_2O_5 excreted.	Total P_2O_5 intake.	Balance per week.	Balance per day.
June 8–14........	528.56	2.71	100.35	631.32	719.62	+ 88.00	+12.57
" 15–21........	484.70	5.63	72.66	562.99	760.09	+197.10	+28.14
" 22–28........	527.94	4.96	71.16	604.06	792.41	+188.35	+26.91
" 29–July 5....	443.72	4.66	72.91	521.29	749.90	+228.61	+32.66

green alfalfa varied from 1.42 to 2.05 per cent and the P_2O_5 from 0.57 to 0.73 per cent.

In Tables II, III, and IV are recorded the data on the income and outgo of calcium and phosphorus for the three animals, re-

spectively; in addition there is added a column of the milk yield for periods of 7 days. It should be noted that there was a decline in milk yield in the case of Animals 2 and 3 after the 5th week of

TABLE III.

Record of Calcium Balance of Animal 2.

Period.	CaO in feces.	CaO in urine.	CaO in milk.	Total CaO excreted.	Total CaO intake.	Balance per week.	Balance per day.	Milk per week.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*lb.*

Dry hay period.

Period.	CaO in feces.	CaO in urine.	CaO in milk.	Total CaO excreted.	Total CaO intake.	Balance per week.	Balance per day.	Milk per week.
May 11–17	619.85	0.60	211.89	832.34	812.01	−20.33	−2.90	288
" 18–24	543.68	0.61	223.58	767.87	812.01	+44.14	+6.30	288
" 25–31	571.20	1.84	219.58	792.62	800.03	+ 7.68	+1.10	284
June 1–7	541.82	0.85	215.24	857.91	800.03	+42.12	+6.02	285

Green hay period.

Period.	CaO in feces.	CaO in urine.	CaO in milk.	Total CaO excreted.	Total CaO intake.	Balance per week.	Balance per day.	Milk per week.
June 8–14	523.80	0.61	197.15	721.56	795.88	+ 74.32	+10.62	261
" 15–21	611.14	0.67	181.30	793.11	856.69	+ 63.58	+ 9.08	237
" 22–28	690.12	0.51	206.58	897.21	1,055.54	+158.33	+22.62	246
" 29–July 5	661.42	0.64	188.98	851.04	882.10	+ 31.06	+ 4.44	227

Record of Phosphorus Balance of Animal 2.

Period.	P_2O_5 in feces.	P_2O_5 in urine.	P_2O_5 in milk.	Total P_2O_5 excreted.	Total P_2O_5 intake.	Balance per week.	Balance per day.

Dry hay period.

Period.	P_2O_5 in feces.	P_2O_5 in urine.	P_2O_5 in milk.	Total P_2O_5 excreted.	Total P_2O_5 intake.	Balance per week.	Balance per day.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
May 11–17	556.42	21.05	239.36	816.83	997.66	+180.83	+25.83
" 18–24	645.09	19.08	235.35	899.52	997.66	+ 98.14	+14.02
" 25–31	625.46	25.02	236.36	886.84	984.31	+ 97.47	+13.92
June 1–7	722.42	21.33	239.87	983.62	984.31	+ 0.69	+ 0.10

Green hay period.

Period.	P_2O_5 in feces.	P_2O_5 in urine.	P_2O_5 in milk.	Total P_2O_5 excreted.	Total P_2O_5 intake.	Balance per week.	Balance per day.
June 8–14	688.50	11.21	217.35	917.06	969.73	+ 52.67	+ 7.52
" 15–21	780.45	6.65	187.75	974.85	1,023.12	+ 48.27	+ 6.89
" 22–28	728.46	4.33	206.46	939.25	1,043.61	+104.36	+14.91
" 29–July 5	704.09	6.42	178.76	889.27	1,018.47	+129.20	+18.47

observation, and in the case of Animal 1 somewhat earlier. This decline we attribute to the very hot weather prevailing at that time and trouble with flies.

TABLE IV.

Record of Calcium Balance of Animal 3.

Period.	CaO in feces.	CaO in urine.	CaO in milk.	Total CaO excreted.	Total CaO intake.	Balance per week.	Balance per day.	Milk per week.
				Dry hay period.				
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*lb.*
May 11–17......	680.44	0.64	136.32	817.40	793.97	−23.43	−3.35	179
" 18–24......	645.89	0.78	140.31	786.98	793.97	+ 6.99	+0.99	175
" 25–31......	629.56	0.58	132.36	762.50	782.26	+19.76	+2.82	171
June. 1–7.......	598.60	0.47	130.23	729.30	782.26	+52.96	+7.56	172
				Green hay period.				
June 8–14......	594.60	0.48	126.28	721.36	777.84	+ 56.48	+ 8.07	169
" 15–21......	602.64	0.92	107.81	711.37	838.65	+127.28	+18.18	139
" 22–28......	708.78	0.67	113.79	823.24	1,037.50	+214.26	+30.61	128
" 29–July 5..	681.72	0.74	102.17	784.63	868.33	+ 83.70	+11.96	128

Record of Phosphorus Balance of Animal 3.

Period.	P_2O_5 in feces.	P_2O_5 in urine.	P_2O_5 in milk.	Total P_2O_5 excreted.	Total P_2O_5 intake.	Balance per week.	Balance per day.
				Dry hay period.			
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
May 11–17........	654.71	22.33	166.46	843.50	823.51	− 19.99	− 2.85
" 18–24........	507.12	12.56	152.23	671.91	823.51	+141.60	+20.23
" 25–31........	629.57	17.68	149.50	796.75	810.16	+ 13.41	+ 1.91
June 1–7..........	646.07	10.55	147.49	804.11	810.16	+ 6.05	+ 0.86
				Green hay period.			
June 8–14........	626.18	3.58	138.60	768.36	795.58	+ 27.22	+ 3.89
" 15–21........	622.17	6.13	112.85	741.15	848.97	+107.82	+15.40
" 22–28........	672.28	5.78	114.98	793.04	869.46	+ 76.42	+10.92
" 29–July 5.....	653.94	6.20	110.35	770.49	846.03	+ 75.54	+10.79

DISCUSSION.

It can be seen from the data in Table II that Animal 1 was in positive calcium and phosphorus balances on both the dry and the green alfalfa, the average daily storage of calcium being, respectively, 14.95 gm. on the dry alfalfa hay and 33.49 gm. on the green alfalfa. This larger storage of calcium on the green alfalfa we would interpret as being in harmony with previous observa-

tions that green plant tissue contains more than dried plant tissue of some vitamine favoring calcium assimilation.

Animals 2 and 3 were also in positive calcium and phosphorus balances in both periods of observation, but not to the same degree as No. 1.   This difference is to be explained by the fact that No. 1 had been receiving poor hay (low in calcium) previous to being put on the experiment, and consequently was in a more depleted condition with respect to the minerals (calcium and phosphorus) than Nos. 2 and 3, which had received alfalfa hay for 20 weeks previous to being placed in this experiment.   In agreement with the results secured with No. 1 there was a greater tendency on the part of these animals to store calcium during the green alfalfa period than during the dry alfalfa period by both Animals 2 and 3.   No. 2 showed a positive daily average calcium balance of but 2.6 gm. during the dry alfalfa period but this was increased to 11.66 gm. in the green alfalfa period.   No. 3 showed an average positive daily calcium balance of 2.00 gm. during the dry alfalfa period and 17.20 gm. during the green alfalfa period.   No. 2 was an especially heavy milker—yielding over 40 pounds of milk per day.

The more pronounced calcium storage observed during the *green* alfalfa period as compared with the *dry* alfalfa period is not to be explained as due to differences in calcium intake alone. While the calcium intake during the green alfalfa period was in some cases higher than during the dry alfalfa period, yet the retention of calcium was so much greater in the former period as to exclude the slight differences in the quantity of calcium ingested as the sole determining factor in the results.

Irrelevant to the direct purpose of this paper but well worth recording was the fact that the milk of No. 2 during the feeding of dry alfalfa hay and also previous thereto, but only while receiving dry hay, was coagulable by heat at 136°, the temperature of a boiling xylene bath.   This coagulability could be prevented by the addition of calcium salts (6).   When this animal was changed to the green alfalfa hay, the milk still retained the property of coagulability by heat at 136°C., but instead of being corrected by the addition of calcium salts, the coagulation point was lowered by such additions and was corrected for by the addition of citrates.

Why these data on calcium balances are at variance with those reported by Forbes and with later results secured by ourselves it is difficult to see. The only suggestion as an explanation that we can offer at the present time is the possibility of a difference in the character of the dry alfalfa hay and silage fed. The dry alfalfa hay which we used may possibly have been richer in the vitamine, assisting calcium assimilation, than the alfalfa used by Forbes in his experiments and the alfalfa used by us in our second series of experiments to be reported upon in a later publication.

With the corn silage the situation may have been similar. The maturity of the corn and the process of ensilaging, like the curing of the alfalfa are variable factors which may modify the nutritive value of the product.

<center>SUMMARY.</center>

1. Liberally milking cows were maintained in positive calcium balance by *dry* alfalfa hay used as the principal roughage and main source of calcium when supplemented with corn silage and a grain mixture. The hay consumption was 10 pounds per individual per day. It was of excellent quality, having been cured under caps.

2. On *fresh green* alfalfa more liberal storage of calcium was observed with these animals than on dry alfalfa hay.

3. With positive calcium balances there were also positive phosphorus balances with the three animals under observation.

4. These results, in reference to calcium equilibrium in milking cows, do not appear to be in harmony with the findings of Forbes and his associates. We have additional data, however, secured recently and to be published in a later paper, but obtained with another alfalfa hay, which also gave us negative calcium balances.

Apparently the question whether positive or negative calcium balances will prevail in liberally milking cows through the use of such an efficient carrier of calcium as alfalfa hay is determined by the quality of the alfalfa hay used. By the term quality, used in this connection, we mean the relative degree of destruction in the curing processes of the unknown factors affecting calcium assimilation.

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A METHOD FOR THE PREPARATION OF CRYSTALLINE OXYHEMOGLOBIN.

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Available methods for the preparation of crystalline oxyhemoglobin may be divided into two groups, in the first of which alcohol or ether, or both, are used as aids to crystallization, and in the second of which the use of these solvents is avoided. Possible objections to the use of alcohol were early pointed out by Hüfner (1), and Mayet (2) emphasized the advantages of "benzine" over ether. Although these workers, as well as Bohr (3) and Torup (4), made preparations in which the use of either or both of these substances was avoided, the first systematic attempt to work out a method without their aid seems to have been the recent one of Dudley and Evans (5). Their procedure involves pressure dialysis of the washed red cells of horse blood, crystallization of the oxyhemoglobin in the dialysate by oxidation, and recrystallization of the product from water by reduction *in vacuo* at 37° and subsequent oxidation. Data as to the yield and purity of the product are not given.

In the hope of obtaining fairly large amounts of oxyhemoglobin in the highest possible state of purity, the Dudley and Evans method was first employed, using a modification suggested by Adolph and Ferry (6); namely, final dialysis against water saturated with carbon dioxide. The oxyhemoglobin obtained in this way crystallized as large plates, instead of the needles reported by Dudley and Evans. Furthermore, the insolubility of the plates and their consequent resistance to reduction not only justified the belief that isoelectric oxyhemoglobin was being dealt with, and that Dudley and Evans were probably working with a more soluble salt, but rendered the use of the method impossible for the purpose in view, and it was abandoned in favor of a different principle.

The method now proposed depends upon observations that suspensions of washed dog or horse red cells crystallize rapidly and almost completely in the presence of toluene when saturated with carbon dioxide and oxygen, and that the resulting oxyhemoglobin may be recrystallized by solution with the aid of sodium carbonate and reprecipitated with carbon dioxide.

The use of toluene was found to hasten markedly the crystallization of the oxyhemoglobin of the corpuscles owing to its disintegrating effect on the cells themselves. While its hemolytic action is slower than that of ether, its use obviates the chief disadvantages of the latter; namely, solubility in water, and the presence of peroxides and other reactive substances which may alter oxyhemoglobin. The carbon dioxide shifts the reaction in the acid direction past the isoelectric point of oxyhemoglobin, so that the crystals obtained are oxyhemoglobin uncombined with alkali. By thus promoting the crystallization of the oxyhemoglobin the acidification also aids in the original disintegration of the corpuscles. Saturation with pure carbon dioxide would, however, drive oxygen out of the solution and change the oxyhemoglobin to the reduced form, which is too soluble to crystallize readily. In order to obviate this difficulty 1 part of oxygen was mixed in a cylinder with 4 parts of carbon dioxide for the saturation. Such a mixture may be passed through oxyhemoglobin solutions indefinitely without reduction.

Removal of the salts is accomplished by the simplified form of pressure dialysis[1] suggested by Adair, Barcroft, and Bock (7), *after* the desired number of recrystallizations has been carried out. Two recrystallizations have been deemed sufficient in this laboratory, but for many purposes the oxyhemoglobin will undoubtedly be found pure enough after the first recrystallization. On the other hand, the losses involved in each recrystallization, while appreciable, are not sufficiently large to preclude three or even four recrystallizations.

Three precautions have been found essential: (a) All operations are carried out in the cold, centrifugation being a possible exception if a centrifuge in a cold room is not available. (b) The oxyhemoglobin is not allowed to become dry, owing to the resultant change, noted by Bohr (8), into a modification in which the

_____
[1] Except that the membranes are not sterilized.

TABLE I.

Preparation No.	Volume of blood.	Total oxyhemoglobin content.	Preliminary separation in centrifuge.	First recrystallisation.			Second recrystallisation.			Yield.		Oxygen capacity.	Conductivity at 25° of saturated aqueous solution.*	Concentration of saturated aqueous solution.
				Volume of H₂O.	Total volume of suspension.	n Na₂CO₃	Volume of H₂O.	Total volume.	n Na₂CO₃					
	$cc.$	$gm.$		$cc.$	$cc.$	$cc.$	$cc.$	$cc.$	$cc.$	$gm.$	per cent	per cent	mhos.	per cent
Dog 8.	150	29.4	+	50	85	10–11	30	65	8.5–9	7.8	26.5	96.3	6.6×10^{-5}	
" 9.	295	55.5	–	50	200	6–6.5	30	80	9–10	13.1	23.6	100.0	—	4.3
" 10.	300	45.9	–	70	160	10–11	30	90	18–19	14.4	31.4	96.3	3.9×10^{-5}	
" 11.	465	76.3	+	100	230	18–22.5	70	170	7	29.2	38.3	99.3	5.4×10^{-5}	
" 12.	248	37.3	–	60	140	10–11	35	80		8.95	24.0	98.0	5.2×10^{-5}	3.9
Horse 6.	605	72.0	–	40	210	15	35	100	5–6	18.75	26.0	97.7	6.6×10^{-5}	2.7

* For the preparation of the solutions for conductivity determinations, see p. 38.

oxygen is not reactive. (c) During the various manipulations on the acid side of the isoelectric point, before the final dialysis, care is taken to have an excess of carbon dioxide constantly present. If the carbon dioxide tension is permitted to fall, part of the oxyhemoglobin is redissolved as alkali salt.

The purity of the oxyhemoglobin obtained by the present method has been controlled by a determination of the ratio of the oxyhemoglobin present, as determined by Van Slyke and Stadie's procedure (9), to the total hemoglobin pigments present, determined as cyanhemoglobin by Stadie's method (10). As will be seen in Table I, preparations of 96 to 100 per cent of the theoretical oxygen capacity were obtained. The relative freedom of the product from salts was controlled by conductivity measurements of saturated aqueous solutions, the values obtained being also given in the table.

EXPERIMENTAL.

Oxalated or defibrinated dog or horse blood of known oxyhemoglobin content is centrifuged and the plasma or serum and the layer of white cells are removed. The red cells are then washed three times with chilled 0.85 per cent sodium chloride solution, after which the supernatant liquid usually gives at most only a faint haze when a test portion is boiled. The cells are then rinsed into a flask with a few cubic centimeters of water. The vessel is cooled in ice water, and a steady stream of a mixture of 4 parts of carbon dioxide to 1 part of oxygen passed in. Toluene is, meanwhile, added in amount equal to about one-seventh of the volume of corpuscles, and the mixture is stirred with the gas inlet tube until it becomes pasty. Passage of the gas is continued for a few minutes, with vigorous stirring, after which the flask is stoppered tightly with a rubber stopper and allowed to stand over night in the ice box. This is often long enough to complete the process of disintegration of the cells and crystallization of the oxyhemoglobin, but if many intact cells are still to be seen under the microscope the treatment with carbon dioxide and oxygen is repeated and the flask allowed to stand a day or two longer.

The consistency of the resulting mixture depends somewhat upon the extent to which the red cells have been packed in the centrifuge and upon other factors which have not been determined.

If the mixture is sufficiently thin it may be centrifuged with advantage in chilled tubes in a cold room, separating into an upper layer of toluene and cell fragments, an intermediate layer of clear solution, and a lower layer of oxyhemoglobin crystals. The two upper layers are poured off and the crystals drained in the ice box on a chilled porous plate, the surface layer being renewed constantly as it dries out, in order to avoid possible conversion of the oxyhemoglobin into a form in which the oxygen is less reactive. During this process a slow stream of carbon dioxide should be directed over the surface of the plate, otherwise a portion of the oxyhemoglobin will redissolve as carbon dioxide evaporates from the mixture. When drainage is as complete as possible, the oxyhemoglobin is scraped into a chilled mortar and ground to a smooth paste with sufficient ice-cold water to bring the final volume up to three to three and a half times (in cubic centimeters) the weight in grams of oxyhemoglobin present in the original blood.

In case the crude mixture of crystals, toluene, and cell fragments is too thick to permit centrifugation, the entire mass is transferred to a porous plate, using the same precautions as given above. Under these conditions the process of drainage takes much longer and cannot be carried to completion owing to the emulsion formed by the toluene. On the other hand, the product, being less compact, is easier to grind to a smooth paste with water, and the toluene and cell fragments may be removed during the first recrystallization. The final volume in this case should be kept as close as possible to that given above.[2]

The thin paste of crude oxyhemoglobin is transferred to a beaker, set in ice water, and titrated to minimum turbidity with normal sodium carbonate solution. During the addition of carbonate the mixture is stirred thoroughly, and any lumps which may remain are disintegrated. The amount of sodium carbonate necessary is greatest, of course, when the crude crystals have been thoroughly drained and contain as little as possible of the bicarbonate and

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[2] An alternative method, which is quite satisfactory in the case of dog blood, but is very slow in the case of horse blood, is to filter the entire mass in the ice box through silk, using as large a Buchner funnel as possible, and observing the precautions given below for filtering oxyhemoglobin suspensions.

salts of the mother liquor. In this case the final concentration of alkali added as carbonate is approximately 0.1 N. If the toluene and cell fragments have been separated previously by centrifugation and if enough water is present, a fairly clear, deep red solution will result, but if too little water is used a crystalline precipitate of what appears to be sodium oxyhemoglobinate will remain. In this case, and also in the case in which the toluene and cell fragments are still present, the carbonate solution is added to the point of minimum turbidity, after which 1 or 2 cc. more are added in order to make sure of an excess.

The solution is next centrifuged, and any toluene and cell fragments on top are sucked off through a capillary tube, a process which can generally be accomplished without appreciable loss of the actual oxyhemoglobin solution. If loss should occur, however, the mixture which has been sucked off may be whirled again and the clear oxyhemoglobin solution added to the main portion. If enough alkali has been added and there is still a crystalline deposit in the centrifuge tubes, too little water is present, and the precipitate may be dissolved in the minimum amount of water and the solution added to the main portion. This precipitate, which is usually encountered at this point only when dog blood has been used. appears to be sodium oxyhemoglobinate, for it is readily soluble in water with a bright red color, it has a characteristic crystalline form, and, finally, yields crystals characteristic of dog oxyhemoglobin when a concentrated aqueous solution is saturated with carbon dioxide-oxygen mixture and allowed to stand in the cold. Further investigation of this salt will be undertaken.

The oxyhemoglobin solution is next chilled and a stream of the carbon dioxide-oxygen mixture passed in until crystallization *begins*, after which the flask is tightly stoppered and set in the ice box. Often within a few minutes the oxyhemoglobin has set to a solid cake of long, flat, scarlet needles in the case of dog oxyhemoglobin, and dark red, glistening, broader plates, often diamond-shaped or hexagonal, in the case of horse oxyhemoglobin.

After standing over night in the ice box the crystals are sucked off on hardened paper in a Buchner funnel (the 5 inch size is adequate for the oxyhemoglobin from 300 cc. of blood). The filtration is carried out in the ice box, with a slow stream of

carbon dioxide passing into the funnel. The surface is kept moist by renewal with a spatula as it dries out, and when this is no longer possible, a few cc. of water saturated with carbon dioxide are sucked through with the same precautions, after which the filtration is stopped. The entire process usually takes less than 1 hour.

For many purposes the oxyhemoglobin is undoubtedly sufficiently pure at this point, and in one experiment which was interrupted at this stage the amount of crystalline oxyhemoglobin recovered was 46 per cent of the amount present in the original blood, as determined by the oxygen capacity.

For further purification the recrystallization process is repeated. The crystalline cake is transferred to a chilled mortar and again ground to a smooth paste with cold water. The volume of the suspension thus obtained should be about 0.7 of that employed for the first recrystallization if the toluene and cell fragments have been initially removed by centrifugation, and from 0.4 to 0.6 as large if the removal of the upper layer was accomplished during the first recrystallization. The larger fraction will, of course, be necessary when drainage of the original crystallized cell mixture on the porous plate has been most complete. The suspension of oxyhemoglobin is dissolved with normal sodium carbonate solution, centrifuged, reprecipitated with the carbon dioxide-oxygen mixture, and collected, at every step with the same precautions as in the first recrystallization.

If salt-free oxyhemoglobin is desired, the crystals are ground with the minimum amount of cold water to a paste which will just flow easily, saturated in the cold with the carbon dioxide-oxygen mixture, transferred at once to narrow collodion dialysis bags, and dialyzed under pressure in the ice box against water saturated with carbon dioxide-oxygen mixture. Dialysis for 3 or 4 days, the carbon dioxide-oxygen-saturated water and the positions of the bags being changed daily, is sufficient to bring the conductivity down to the values given in Table I. The dialysis tubes found most suitable in this laboratory were made in 50 cc. test-tubes with one of the eminently satisfactory collodion mixtures proposed by Eggerth (11), namely, a solution of 7 gm. of "Parlodion" in 60 cc. of ether, 30 cc. of alcohol, and 10 cc. of glacial acetic acid. Dialysis under pressure was accomplished by simply

closing the ends of the tubes with tightly screwed, rubber-faced screw pinch-cocks, as proposed by Adair, Barcroft, and Bock (7).

At the end of the dialysis the contents of the bags, which still retain their crystalline structure, are sucked off in the ice box on hardened paper in a Buchner funnel, using, as before, the precaution of keeping the surface layer moist. The use of carbon dioxide at this stage is unnecessary, as the oxyhemoglobin remains sparingly soluble in the absence of alkali and salts.

The conductivity values were obtained by grinding the product in a chilled mortar with ice water, centrifuging the resulting suspension, pouring off at temperatures ranging from 19 to 28°, and measuring the conductivity of the clear supernatant solution after evacuating a few times to remove any carbon dioxide present. The oxyhemoglobin content of the resulting solutions is given in Table I.

The purified oxyhemoglobin was dissolved either with the aid of sodium carbonate solution or with a sufficient excess of N/7 sodium hydroxide to bring the final concentration of alkali to 0.03 or 0.04 N, and the solution was filtered through a small, loose plug of washed cotton into a volumetric flask of appropriate size and made up to the mark. The yield of oxyhemoglobin was calculated from the oxygen capacity of the resulting solution, and varied between 23 and 38 per cent of the total originally present in the blood used. The purity of the product was determined by comparing the oxygen capacity with the total hemoglobin content as determined by Stadie's (10) methemoglobin method,[3] and the ratio of oxyhemoglobin to total hemoglobin pigments was found to vary between 96 and 100 per cent.[4]

The entire process of preparation of the dialyzed oxyhemoglobin can scarcely be completed in less than a week. On the other hand, it is not desirable to let preparations stand unduly long at the various stages of purification, for if the process is extended

[3] It is advisable to check up the cyanhemoglobin standard at least every 2 weeks, as the color tends to deepen, even in the ice box. The color of the chilled standard also changes with rise in temperature, so that the solution should be allowed to come to room temperature before comparisons are made.

[4] The experiments recorded in this paper were all performed before the hot weather set in. Since that time yields and oxygen capacities have occasionally dropped as much as 5 per cent below the values given above.

for much over 2 weeks there is a noticeable diminution of the oxygen-binding power of the product. Also, when solutions saturated with the carbon dioxide-oxygen mixture are allowed to stand it is desirable to resaturate with the gas mixture at least every other day in order to compensate for leakage.

The stability to be expected of the solutions of oxyhemoglobin obtained by this method is indicated in Fig. 1, in which a steady diminution of the oxyhemoglobin content is shown amounting roughly to 1 per cent per day. The total hemoglobin content
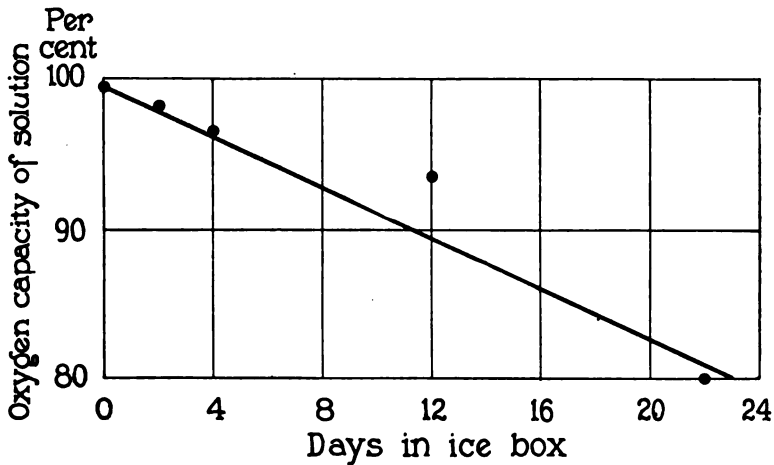
FIG. 1.

of the original solution was 13.8 gm. per 100 cc. and the oxyhemoglobin content 13.7 gm., while the latter had fallen to 11.05 gm. at the end of 22 days.

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GROWTH AND REPRODUCTION UPON SIMPLIFIED FOOD SUPPLY.

II. INFLUENCE OF FOOD UPON MOTHER AND YOUNG DURING THE LACTATION PERIOD.[*]

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In the preceding paper of this series[1] experiments were described in which it appeared that the quantitative proportion of milk in diets consisting of ground whole wheat and whole milk powder had a distinct influence upon the mother and young during the lactation period even in cases in which both diets would be accepted as adequate since both sufficed for growth, reproduction, and successful suckling of the young.

Thus two female rats, whose diets contained, respectively, one-sixth and one-third of milk solids, both raised good sized litters of young, but the larger proportion of milk in the diet resulted in more rapid growth of the young and less loss of weight on the part of the mother while suckling them.

Since, in our experience, individual variability constitutes an even more prominent factor in reproduction and lactation than in growth, we have extended the comparison of the efficiencies of these two diets for the support of lactation in the mother and growth in the suckling young to a large number of cases the average results of which are presented briefly in this paper.

For the convenience of the reader the diets used in the particular series of experiments with which this and the following paper[2]

[1] Sherman, H. C., Rouse, M. E., Allen, B., and Woods, E., *J. Biol. Chem.*, 1921, xlvi, 503.

[2] Sherman, H. C., and Crocker, J., *J. Biol. Chem.*, 1922, liii, 49.

deal have been given alphabetical designations in order of in-
creasing proportions of milk in the food mixture.

Diet A (Laboratory No. 16) consists of one-sixth whole milk
powder and five-sixths ground whole wheat with sodium chloride
2 per cent of the weight of the wheat.

Diet B (Laboratory No. 13) consists of one-third whole milk
powder and two-thirds ground whole wheat with sodium chloride
2 per cent of the weight of the wheat.

Only distilled water was given and no bedding was used except
in the cases of females with young less than 2 weeks old, and then
only pure paper or cellulose.   The rats had no access to wood
or any other material which could have been eaten in addition
to the regular diet.   The diet was fed *ad libitum*, the ingredients
being so ground and mixed that the animals were obliged to
consume them in the exact proportions stated, and the amount of
food consumed was carefully determined and recorded in periods
of 1 week.

The general procedure was as follows.   Rats breeding on experi-
mental diets are kept in galvanized wire cages in lots consisting
usually of one male and three to five females.   Each rat is weighed
weekly at all times and more frequently as pregnancy advances.
Each female when apparently within a few days of giving birth
to young, is placed in a separate cage.   As soon as the young are
born a new record of the mother's food consumption is begun and
the weighings of mother, young, and food are then made at
weekly intervals until the young are 4 weeks old when they are
separated from the mother and the latter is returned to her breed-
ing cage.   Living in bare cages, our rats practically lose the nest-
making instinct, and even if offered bedding do not make nests in
advance of the birth of young.   As a regular procedure we do
not offer bedding until after the birth of a litter is complete.
Some hours after the young have been born on the bare wire
cloth bottom of the cage, bedding is supplied in the form of
finely cut pure crêpe paper or pure cellulose in shredded form or
a mixture of these.   The mother may or may not make a rough
temporary nest of this material.   The cages are cleaned and fresh
bedding is supplied daily.   This involves frequent but uniform
handling of the young after they are 24 to 36 hours old.

Doubtless the technique which we have adopted in order to insure cleanliness of cages, complete recovery of scattered food, and avoidance of any form of bedding which might be eaten by the rats, is somewhat severe and results in the rearing of a somewhat lower percentage of the young than might be reared by the same mothers on the same diets under the less rigorous conditions adopted in some other laboratories. Even if it be thought that our endeavor to insure a strictly quantitative record of food intake and the absence of roughage other than that supplied by the food, has resulted in unduly rigorous conditions and a consequently high rate of infant mortality, yet since these conditions are all uniform they cannot detract from the accuracy of our comparisons of different diets; and they do permit of a more strictly quantitative discussion of the efficiencies of different diets than would otherwise be possible.

TABLE I.

Comparison of Numbers of Young Born, and of Young Reared, by Ten Mothers on Each of Two Diets, A and B.

Diet.	Number of young born.		Number of young reared.		Percentage of young reared.
	Total.	Average.	Total.	Average.	
A	299	29.9	145	14.5	48
B	498	49.8	310	31.0	62

Influence of Diet upon Numbers of Young Born and Successfully Suckled.—The effects of the two diets here considered is best shown by a comparison of the complete reproduction records of the first ten females kept on each of the two diets, as shown in Table I.

Here the increased proportion of milk in the diet resulted in the birth of a larger number of young and also the rearing of a larger percentage of the young born.

A similar relationship was found when we compared the data of all the births during the year 1920; *viz.*, 167 litters born on Diet A and 402 litters born on Diet B. These latter figures are not here given in detail because their discussion is rendered somewhat cumbersome by the fact that both of these latter groups contain a large proportion of young mothers whose tendency to lose their first litters makes the percentage of infant mortality in

the averages for the year somewhat misleading unless explained in detail.   It may, however, be emphasized that an experience with hundreds of litters born at all seasons of the year has fully confirmed the conclusions drawn from the data shown in Table I.

In this larger experience of 167 litters on Diet A and 402 litters on Diet B the increased proportion of milk in the diet of the mother evidently resulted both in the bearing and rearing of more young and in an increase in the number of young born per litter.   On Diet A the most frequent number was six and the mean number was 5.51; on Diet B the most frequent number was seven and the mean was 6.47.   As the difference between the means was over seven times its probable error, it is undoubtedly significant.   A similar difference was found in the number of young raised per litter.   As the number of litters raised by each mother was much larger among those receiving the higher proportion of milk in the diet, the net effect of this change in the quantitative proportions of foods in the dietary was the rearing of about double the number of young by each mother as illustrated by the typical data given in Table I.

Influence of Diet upon Maintenance of Mothers' Weight while Suckling Young.—As mentioned above, our technique involves weekly weighings of all rats in our colony.   Hence by referring back we can always find the weight of each female at approximately the beginning of her pregnancy, 3 weeks before the birth of her young.   With this weight we have compared the lowest weight observed during the lactation period as an indication of the efficiency of the diet in maintaining the mother while she is suckling her young.

When the number of young in the litter was small, and the demands of lactation relatively light, either of the diets here discussed was adequate for maintenance of the body weight of the mother; but with larger litters and consequently larger demands upon the suckling mothers, the diet containing the larger proportion of milk proved much more efficient in the maintenance of the body weight of the mother.   Averaging for each diet all of the cases of mothers suckling litters of six young, the net difference in favor of Diet B was 12 gm. per capita or over 6 per cent of the mother's weight.   For those suckling seven young the average difference was 35 gm. per capita or 18 per cent of the

mother's weight. For larger litters there are not sufficient numbers of cases of mothers of similar ages to permit of quantitative comparison.

It is plain that, when the demands of lactation are considerable, the quantitative proportions of milk in the two diets (each of which would ordinarily be adjudged adequate) becomes an important consideration in the maintenance of the mother as well as in the rearing of the young.

Influence of Diet of Mother upon Growth of Suckling Young.— In the experiments here described we did not weigh the young at birth but did weigh them at weekly intervals thereafter. From previous experience of our own and other laboratories we judge that healthy rats will vary but little from a range of 4 to 5 gm. at birth. At 4 weeks of age, however, our young rats of families on Diet A averaged 33.9 gm. (± 0.3 gm.), and those of families on Diet B averaged 42.3 gm. (±0.2 gm.). This increase of 8.4 gm. (±0.4 gm.), or 25 per cent in the average weight at weaning time, is far too large to be accidental and is plainly due to the increased percentage of milk in the food of the mother. In Table II the data are grouped according to the number of young in the litter. Since practically equal numbers of males and females were reared on each diet it is not necessary in this comparison to discuss the sexes separately.

It is evident from Table II that Diet A which appears fully adequate under ordinary tests fails to permit of a fully average growth of the suckling young especially in the larger families, whereas Diet B resulted in a fully average weight at weaning time even when the numbers of young were large.

Relation of the Proportion of Milk in the Diet to the Economy with Which the Food Is Used.— The foregoing data show that more and larger young were weaned with less drain upon the mother when the food of the family contained the higher percentage of milk. It would be of interest to know to what extent the better results are attributable to the consumption of more as well as better food, and to what extent to a more economical use of the food consumed. Too many factors enter into this problem to permit of our classifying all of our cases into comparisons in which only one factor shall vary at a time. This condition is, however, approximated in the case of the groups of

mothers raising litters of five, six, and seven young on the two
diets, respectively. These groups of females were of nearly
the same average initial weight and had nearly, if not quite,
completed their growth. From records of food consumption of
large numbers of normal rats in our colony it appears that young
adults require an average of 0.22 calories per gm. of body weight
per day for their own support. Making this allowance for the
maintenance of the mother we may calculate the rest of the food
actually consumed as chargeable to the rearing of the young.
(In the latter part of the 4 weeks period during which the young
remain with the mother, a part of the food furnished is, of course,

TABLE II.

Influence of Diet of Family on Size of Young at Weaning.

Number of young in litter.	Diet A.		Diet B.		Difference in favor of Diet B at 28 days.	
	Total number of young.	Average weight at 28 days.	Total number of young.	Average weight at 28 days.		
		gm.		*gm.*	*gm.*	*per cent*
2	4	38	4	43	+ 5	+13
3	12	36	15	43	+ 7	+19
4	36	38	32	40	+ 2	+ 5
5	65	34	80	41	+ 7	+21
6	66	35	132	41	+ 6	+17
7	35	34	154	44	+10	+29
8	16	26	88	43	+17	+65
9	0		45	41	·	
10	10	28	10	43	+15	+54

consumed directly by the young. For the comparisons which
we are here making we need not consider whether the food which
goes to nourish the mother and young is consumed in the first place
entirely by the mother or in part by her and in part by the
young directly.)

Taking then the records of food consumption of the three
most directly comparable groups of families on each of the two
diets, namely those having litters of five, six, and seven young,
deducting what the mothers would normally have eaten for their
own maintenance, and disregarding for the moment the changes
in body weight of the mother which actually occurred, it appears

that each gram of young rat reared to weaning time on Diet A cost 7.30 calories of extra food, while on Diet B this extra food cost was 6.74 calories. As these are averages for 27 families on Diet A and 60 families on Diet B and the difference appears consistently when the groups having five, six, and seven young are compared separately, the result cannot be accidental. It plainly establishes a more economical use of the diet containing the larger percentage of milk. The true difference in favor of this diet is seen to be considerably greater when account is taken of the fact that the body weight of the mother was at the same time much better supported by Diet B than by Diet A, the average difference in the cases here considered being 14.5 gm. in favor of Diet B for each nursing mother, or 7 to 8 per cent of her body weight. Hence it appears that the young rats of the families receiving the larger percentage of milk in their food were produced with greater economy both of the body material of the mother and of the calories of food consumed.

SUMMARY.

Breeding rats were fed upon diets containing respectively one-sixth whole milk powder to five-sixths ground whole wheat or one-third whole milk powder to two-thirds ground whole wheat. Young were successfully reared on both diets and both would be regarded as adequate for growth, reproduction, and successful suckling of the second generation. The larger proportion of milk in the second diet resulted in the following evidences of improved nutrition:

1. Increase in the number of young produced.

2. Increase in the percentage (and therefore also in the number) of young successfully suckled.

3. Better maintenance of the body weight by the mother while suckling the young.

4. Higher average weight of young at a standard weaning age of 4 weeks.

5. More economical utilization of the calories of food consumed (as well as of the body material of the mother) in the rearing of the young to weaning age.

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GROWTH AND REPRODUCTION UPON SIMPLIFIED FOOD SUPPLY.

III. THE EFFICIENCY OF GROWTH AS INFLUENCED BY THE PROPORTION OF MILK IN THE DIET.[*]

By H. C. SHERMAN and JOSEPHINE CROCKER.

(From the Department of Chemistry, Columbia University, New York.)

In the preceding papers of this series [1,2] it has been shown that food mixtures consisting of ground whole wheat and whole milk powder with distilled water and sodium chloride, are adequate for growth and reproduction in our experimental animals (rats) when one-sixth of the weight of the food mixture is milk powder, but that an increase in the proportion of milk to one-third of the solids of the food mixture results in more efficient nutrition as evidenced in better maintenance of the mother during the lactation period and the production and successful suckling of more and larger young.

The purpose of the present paper is to extend this study of the influence of the quantitative proportions of these simple mixtures by following their effects upon the rate and efficiency of growth of the young after weaning, and by considering also the effects of diets containing larger proportions of milk.

The series of diets of whole wheat and whole milk powder here referred to is as follows:

Diet A (Laboratory No. 16).—One-sixth whole milk powder and five-sixths ground whole wheat with sodium chloride 2 per cent of the weight of the wheat. Energy value 3.79 calories per gm.

Diet B (Laboratory No. 13).—One-third whole milk powder and

[1] Sherman, H. C., Rouse, M. E., Allen, B., and Woods, E., *J. Biol. Chem.,* 1921, xlvi, 503.

[2] Sherman, H. C., and Muhlfeld, M., *J. Biol. Chem.,* 1922, liii, 41.

two-thirds ground whole wheat with sodium chloride 2 per cent of the weight of the wheat.   Energy value 4.04 calories per gm.

Diet C (Laboratory No. 73).—Equal weights of whole milk powder and ground whole wheat with sodium chloride 2 per cent of the weight of the wheat.   Energy value 4.29 calories per gm.

Diet D (Laboratory No. 80).—Two-thirds whole milk powder and one-third ground whole wheat with sodium chloride 2 per cent of the weight of the wheat.   Energy value 4.55 calories per gm.

For reasons described in the preceding paper,[2] no bedding was used and only distilled water was supplied.

The animals were allowed to eat *ad libitum* from weighed portions of their respective food mixtures and the quantity of food consumed was in each case determined by weighing the remaining food at the same time with the weekly weighing of the rats.

The young rats were separated from their mothers and placed upon the experimental diets at a standard "weaning" age of 4 weeks and thereafter weighed on a regular weekly weighing day.   In the present discussion the weekly records are here combined into a period of 4 weeks, the 5th to 8th weeks (inclusive) of the life of the rat.   The average number of grams of body weight gained per 1,000 calories of food consumed during this definite 4 week period in the life of the rat is found to be a serviceable method of comparing the efficiencies of the diets tested in meeting one phase of nutritive requirement.

In presenting here the net results of this study we shall give only the data which represent the averages of all comparable records available at the time these calculations were made.   We have, however, verified the conclusions thus reached by making considerable numbers of experiments in which carefully matched parallel lots of young animals were placed simultaneously upon the different diets and kept side by side under exactly the same conditions throughout.

The results of comparisons of general averages of all our data and those obtained by comparing smaller numbers of more closely matched animals agree.

Considering it best, therefore, to give the net results of all our experience rather than take space for special comparisons of matched individuals or lots, it becomes necessary to present averages for the different diets in which the ratios of the sexes

among the experimental animals were not kept uniform. It is
well known that the males grow somewhat faster than the females,
but in our experience they eat correspondingly more so that the
comparisons of the efficiencies of the different diets, which is the
problem with which we are here concerned, may be made by
observations either on males, females, or mixed lots of varying
sex ratios. This is illustrated by the following comparison of
two mixed lots of widely different sex ratios, both fed upon Diet B.

Lot 1247 consisting of one male and six females consumed
34 calories per rat per day and gained 71 gm. of body weight per
1,000 calories consumed; while Lot 1261 consisting of six males
and one female consumed 40 calories per rat per day and gained
68 gm. of body weight per 1,000 calories consumed. The agree-
ment between the figures representing the efficiency of the food

TABLE I.

Efficiency of Growth on Different Diets.

Diet.	Number of lots.	Number of rats.	Average gain in gm. per 1.000 calories of food eaten.
A	32	163	54 ± 0.6
B	39	164	73 ± 0.8
C	34	164	74 ± 1.1
D	30	129	76 ± 1.1

as here considered (71 and 68, respectively) is as close as could
be expected from this number of cases, if the sex ratios had been
the same or if all the experimental animals had been of the same
sex. Hence in Table I we present average data without distinc-
tion as to sex. Table I shows general average results for Diets
A, B, C, and D.

It will be seen that Diet B showed marked superiority to Diet
A as regards the efficiency with which the young rats grew
upon these two diets, respectively. The difference per 1,000
calories consumed is here 19 gm. (± 1.0 gm.), a difference which
is certainly significant.

Whether efficiency of growth, as judged by this criterion, can
be still further promoted by increasing the proportion of milk
beyond that of Diet B cannot be stated with entire certainty.
The average gain per 1,000 calories is higher for Diet C than for

Diet B, and higher for Diet D than for Diet C; but the differences in these latter cases are only about as large as their probable errors.

Apparently the diets in which milk furnished from one-third to two-thirds of the total solid matter of the food were for these conditions about equally efficient in supporting rapid growth, and certainly they were much more efficient than the diet composed of the same articles of food in which the proportion of milk was lower.

SUMMARY.

The efficiency of growth as influenced by diet is here studied by determining and comparing the gains in weight per 1,000 calories of food consumed during a fixed period of rapid growth in young rats.

Four diets, composed of whole milk powder and ground whole wheat in different proportions, were compared.   Each diet was tested from 30 to 39 times and upon from 129 to 164 rats.

Diet A in which milk furnishes one-sixth of the solids or one-fifth of the calories of the food has been shown to be adequate; but growth is here shown to be much more efficient when the proportion of milk is increased so that, as in Diets B, C, and D, the milk furnishes from one-third to two-thirds of the solid matter, or 40 to 70 per cent of the total calories, of the food supply.

The method promises to be useful as a further means of studying and comparing the nutritive values of different adequate dietaries.

THE ESTIMATION OF LIPOID PHOSPHORIC ACID ("LECITHIN") IN BLOOD BY APPLICATION OF THE BELL AND DOISY METHOD FOR PHOSPHORUS.

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Because of the comparative simplicity of the Bell and Doisy (1) method for the determination of phosphorus, it seemed desirable to apply this method to the determination of lipoid phosphoric acid ("lecithin") in blood. The nephelometric method of Bloor (2), while accurate and satisfactory in most respects has the disadvantage of requiring considerable time, as well as very careful technique. By adopting Bloor's (2) procedure for preparing and evaporating the alcohol-ether blood extract and the Bell and Doisy (1) method of digestion and color development, it appeared that a much simpler procedure would result.

A few trials of the new method on a composite sample of alcohol-ether extracts of blood sufficed to indicate not only the time-saving advantage of the procedure but, in addition, brought to light several sources of error in the colorimetric method which do not appear to have been previously observed in detail. The first difficulty appeared in trying to obtain check results on the same sample of blood extract. While the results agreed within 10 to 15 per cent, there seemed in some cases to be a poor match between the color of the unknown and that of the standard. This difficulty was thought to be connected with an excess acidity during the first stage of color development (as evidenced by excessive foaming on addition of the alkaline sulfite solution). Consequently, several experiments were made using standard solutions containing the same amount of phosphate solution but varying amounts of acid. As a result it was found that while the difference in acidity was not entirely responsible for the poor

match it was in all probability the explanation of our inability to obtain check results. Table I shows the effect of varying acidity on the color intensity.

From Table I it is apparent that the color in Flasks 1 to 11 decreases with additional quantities of sulfuric acid. It is of interest to note that within the range of acidity represented in Flasks 5 to 8 the effect upon the color development is not so decided as is the case outside of this range. This fact serves to indicate the advisability of using 6 drops of acid in the digestion rather than more or less and, furthermore,

TABLE I.

Effect of Varying Acidity on Depth of Color.

Flask No.	H_3PO_4 present.	Concentrated H_2SO_4.	Colorimeter reading.	Value found.	Error.
	mg.	*drops*	*mm.*	*mg.*	*per cent*
1	0.15	0	17.3	0.173	+15.3
2	0.15	1	18.2	0.165	+10.0
3	0.15	2	18.6	0.161	+ 7.3
4	0.15	3	19.4	0.154	+ 2.7
5	0.15	4	19.7	0.152	+ 1.3
6	0.15	5	19.9	0.151	+ 0.7
7	0.15	6	20.0	0.150	0.0
8	0.15	7	20.3	0.148	− 1.3
9	0.15	8	20.8	0.144	− 4.0
10	0.15	9	21.7	0.138	− 8.0
11	0.15	10	22.2	0.135	−10.0

Color developed in each case by the addition of 2 cc. each of molybdic acid solution and hydroquinone solution, and after standing 5 minutes followed by 10 cc. of carbonate sulfite solution. All were allowed to stand 5 minutes, made up to 25 cc. volume, and compared with No. 7.

shows the importance of carrying the digestion process always to as near the same point as possible. By thus keeping the number of drops of sulfuric acid in the tube after digestion within 4 to 6 drops and adding 6 drops to the standard the error due to acidity falls within experimental limits.

Bell and Doisy (1) state that the color of the solution does not fade at the same rate in the cups of the colorimeter as in the flask. We studied, therefore, the effect of artificial light both during and after color development, and at first were led to believe that strong light intensified the color. If both cups of the colorim-

eter were filled at the same time with the same solution and set at the same height they were found to match. If, however, the cup on the right were removed, filled with a fresh solution from the flask, replaced in the colorimeter, and read immediately, the new sample was found to appear decidedly weaker in color. Furthermore, the new sample seemed to have a slightly different color, a greenish yellow tinge. But after standing for 5 minutes in the colorimeter the colors matched perfectly as regards both quality and intensity. That this result, however, was not due to the effect of light was later shown by repeating the experiment, and allowing the fresh solution to stand for 5 minutes in the dark before reading. It was found to match the other solution which had been exposed to the light. The fresh sample was then poured into a clean dry cup, poured back again, and read. The reading was again about 25 per cent high. The light was turned off for 5 minutes, readings were again made, and once more the colors matched. This was repeated a number of times, always with the same result; *i.e.*, that pouring the solution from one container to another caused the color to appear weak in the colorimeter. This difference in color always disappeared, however, in 4 or 5 minutes. This interesting phenomenon appears to be due to the presence of bubbles in the solution which require about 5 minutes to "settle." It is especially noticeable when the solution has contained considerable acid before neutralizing and for that reason the bubbles are thought to be of carbon dioxide although no attempt has been made to establish definitely this point. The poor match encountered in studying the effect of acidity, and referred to above, was entirely due to the presence of these bubbles in the freshly poured-out solutions. To remedy the matter and avoid error, the standard should be poured back, and the cup refilled each time the solution in the "unknown" cup is changed; or if time permits 5 minutes may be allowed for the fresh solution to "settle" in which case it will not be necessary to disturb the standard.

Myers and Shevky (3) in their recent paper state that the Bell and Doisy method (1) will give accurate results when the standard solution does not contain more than 0.25 mg. of phosphorus per 100 cc. more than the unknown. This conclusion was reached from comparing solutions that had contained only the acid

necessary for the color reaction. In using solutions that have an excess acidity during color development the range of accuracy seems to be still narrower. It has been our experience that in cases where the reading of the unknown is more than 25 per cent above or below that of the standard a stronger or weaker standard should be used. The required standard may be prepared after readings are started if necessary, as the color does not change for at least ½ hour.

Method.

Reagents.—The reagents used are those described by Bell and Doisy (1).

Procedure.—5 cc. of whole blood or plasma are pipetted slowly into about 75 cc. of alcohol-ether mixture (consisting of 3 parts of alcohol and 1 part of ether, both redistilled) contained in a 100 cc. volumetric flask. The flask should be shaken during the addition to avoid the formation of large clots of the precipitate. It is then immersed in boiling water and shaken well to avoid overheating. As soon as the contents begin to boil the flask is removed, the mixture cooled to room temperature, made up to the mark with the alcohol-ether mixture, and filtered. The filtrate may be preserved in a well stoppered bottle for a considerable period without deterioration.

For determination, 10 cc. of whole blood extract or 15 cc. of plasma extract are measured into a large Pyrex digestion tube (25 × 200 mm.), calibrated at 25 cc. and containing three glass beads. The extract is then evaporated to dryness in a boiling water bath. Unless the water bath is cold before immersing the tubes care must be taken to shake the tubes gently until boiling begins, to prevent the extract from boiling over. To the dry residue in the tube 6 drops of concentrated sulfuric acid and 1 cc. of concentrated nitric acid are added. Both acids must, of course, be free from phosphorus. The mixture is then digested with a micro burner, at first over a low flame, then over a higher flame until the nitric acid is driven off and the remaining sulfuric acid is perfectly clear. This digestion usually requires about 10 minutes. After cooling for 1 or 2 minutes the sides of the tube are washed with about 5 cc. of distilled water and 2 cc. each of the molybdic acid solution and the hydroquinone solution

are added.   After mixing and allowing to stand for 5 minutes 10 cc. of the alkaline sulfite solution are added and the whole is well mixed.   After 5 minutes it is made up to the 25 cc. mark with distilled water and compared in the colorimeter with a standard made up as follows: 5 cc. of the standard monopotassium phosphate (containing 0.03 mg. of phosphoric acid per cc.) are added to a 25 cc. volumetric flask or tube graduated at 25 cc., containing 6 drops of concentrated sulfuric acid.   The color is developed in the same manner as described for the unknown.   In making readings with the colorimeter it is necessary to empty the standard cup into the flask and refill it each time the solution in the other cup is changed.   Otherwise, the solution in the unknown cup must be allowed to stand for 5 minutes after being poured out before a reading is made.

If the standard is set at 20 mm. the calculation will be made according to the following formula:

$$S \times \frac{20}{R} \times \frac{20}{X} \times 100 = \text{mg. } H_3PO_4 \text{ per 100 cc. blood}$$

where S equals mg. of H_3PO_4 in amount of standard used, R equals reading of unknown, and X equals cc. of extract used.

Notes on the Method.—It was found that in order to get the best results the method of procedure should be standardized even to the smallest details.   In the digestion, for example, it is advisable always to use the same number of glass beads and to carry the digestion to as near the same point as possible.   For this purpose a tube of the same size as used in the digestion and containing the same number of beads and 6 drops of concentrated sulfuric acid may be set up beside the digestion tubes for comparison.   This tube if calibrated at 25 cc. may be used for the development of the standard.

If a blood containing a normal amount of "lecithin" is being analyzed the standard may be made from 5 cc. of the standard phosphate solution described above.   This should contain 0.15 mg. of phosphoric acid and would, therefore, cover the range 25 to 40 mg. of phosphoric acid per 100 cc. of blood.   A weaker or stronger standard may be prepared very easily, however, if the unknown is found not to fall within the range of the prepared standard.

For the purpose of checking the accuracy of the method a number of determinations were made using both the new colorimetric method and the Bloor nephelometric method on the same samples of human, dog, and rabbit blood. The results were found to agree within 5 per cent as shown in Table II.

The advantages of this method as applied to the determination of "lecithin" may be briefly summarized. The chief advantage is the time saved. In making a large number of determinations it is convenient to run about four digestions at a time. If this is

TABLE II.

Lipoid Phosphoric Acid per 100 Cc.

Specimen.	Colorimeter method.	Bloor method.
	mg.	*mg.*
Human blood...................................	39.25	40.5
" "	40.0	39.0
" " plasma...........................	25.75	26.37
Rabbit blood..................................	78.0	82.0
" " plasma...........................	55.6	55.0
Dog blood.....................................	61.6	64.8
" "	48.3	49.0
" " plasma.........................	54.0	53.5
" " "	37.7	35.5
Composite sample of human, dog, and rabbit blood and plasma...........................	49.3	49.06

done another series of four may be evaporating to dryness while the determination is being carried out on the first four. In this way determinations may be made at the rate of at least one every 10 minutes since 40 minutes is ample time for the digestion, color development, and reading of a series of four samples. The digestion time is cut to 10 minutes as compared with about 25 to 30 minutes in the Bloor method. The titration step in the latter method is eliminated. Furthermore, the use of calibrated tubes for the digestion makes it possible to carry out the whole operation in the same tube, and save the time consumed in transferring solutions from tubes to flasks with possible danger of loss and contamination.

SUMMARY.

1. Varying the acidity during color development is found to have a marked effect.

2. The color is not affected by intense light but appears to be sensitive to mechanical agitation.

3. A procedure is described for applying the Bell and Doisy method to the estimation of lipoid phosphoric acid in blood.

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BUFFER SYSTEMS OF BLOOD SERUM.

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Loaned and Self-Possessed Serum Buffers.

In a previous paper (Doisy and Eaton, 1921), we have shown that the loss of hydrochloric acid from blood serum, by its migration into the cells, approximately accounts for the increase in the binding power of the serum for carbon dioxide when blood is exposed to increased tensions of that gas (Reaction 1). We considered that other acid radicals probably shift in a similar manner, but were unable to obtain any evidence of the passage of base across the cell membrane. Our work was incomplete in that we measured only the buffers loaned to the serum by the cells, and did not attempt to measure the "self-possessed" buffer value of the serum. Of the two types of reaction (Van Slyke, 1921),

(1) $BA + H_2CO_3 \rightarrow BHCO_3 + (HA) \rightarrow$ cells.
(2) B protein $+ H_2CO_3 \rightarrow BHCO_3 + H$ protein.
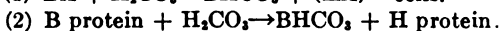
our earlier experiments furnished data only for the first. In order to evaluate the relative importance of each of these buffer reactions, we have extended our work by a further study of the blood of four dogs and of two men.

As was pointed out in our previous paper, the corpuscles of blood occupy a larger volume as the tension of carbon dioxide in the gas with which they are in equilibrium is increased. Although this phenomenon was noted some time ago (von Limbeck, 1894–95), and its importance emphasized in our former paper,

it has not been considered in other recent investigations.[1] For this reason it seemed probable to us that other estimates (Van Slyke and Cullen, 1917; Fridericia, 1920) of the extent of the migration of hydrochloric acid into the cells were too low. The use of anticoagulants is still another reason for thinking that the values previously reported for chloride shift were too low. Our work has made it seem probable that all of the potential acids of the serum take part in the migration, and that they enter into this reaction to a degree which is dependent upon their concentration and possibly upon their relative acid dissociation constants. Samples of oxalated blood generally show that less than 50 per cent of the increase of bicarbonate of serum (on treatment of oxalated whole blood with increasing tensions of CO_2) is due to a loss of chloride, whereas our results on defibrinated blood showed the chloride shift to account much more closely for the gain in bicarbonate. It therefore seems fair to suppose that a shift of oxalic acid explains the difference in behavior, and if this is true it becomes important to avoid all such additions to blood in such studies of acid-base equilibria.

In the experiments here reported we have attempted to analyze the sources of alkali by which the blood combines with increasing amounts of CO_2 and to estimate the quantitative contribution of each. Within a given range of pH, how much of the base for the increase in serum bicarbonate is made available by the migration of acids into the cells (reaction of Type 1, above)? Is the migration of HCl alone sufficient to account for this quota, or do the acids of other salts also migrate? How much base is furnished from the self-possessed, non-migrating buffers of the serum, and to what relative extents do the serum proteins and the serum phosphates participate?

The plan of our experiments was to determine the gain in serum bicarbonate on equilibrating fully oxygenated, defibrinated blood

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[1] L. J. Henderson has discussed this point rather briefly in some of his recent papers (McLean, F. C., Murray, H. A., Jr., and Henderson, L. J., *Proc. Soc. Exp. Biol. and Med.*, 1919-20, xvii, 181; Henderson L. J., *J. Biol. Chem.*, 1920, xli, 427), but apparently has not attempted to make the corrections for change in corpuscle volume as described in this paper. The mechanical models of Henderson and Spiro (Spiro, K., and Henderson, L. J., *Biochem. Z.*, 1909, xv, 114) are very illuminating.

with 20, 40, and 60 mm. tension of CO_2. By determining also the
gain in bicarbonate of separated sera similarly equilibrated with
increasing tensions of CO_2, there is obtained the effect of the self-
possessed serum buffers (Type 2). As indicated by Van Slyke in
his treatment of the data on Joffe's blood, the difference between
the two sets of values (for true serum and separated serum)
represents the buffer action of Type 1, which is due to the pres-
ence of the corpuscles. The determination of chlorides in the true
sera shows the amount of base liberated from NaCl by the migra-
tion of HCl. Since the earlier experiments gave no evidence of
migration of base, by a comparison of the molar gain of bicarbon-
ate due to "loaned buffer" with the molar loss of chloride in true
sera, we attempt to determine the extent to which other acids
migrate and thus contribute to the loaned buffer action. For
one blood we determined also the hemoglobin and inorganic
phosphates of the cells, which data allow the calculation of the
relative amount of base furnished by each to the migrating acids.

The sera were also analyzed for inorganic phosphates and from
this one may calculate the relative amount of base furnished by
phosphate and protein buffers, the self-possessed buffers of the
serum.

EXPERIMENTAL PROCEDURE.

The dog's blood for our experiments was drawn from the femoral
artery while blood from the arm vein was used in the case of the
two men. In each experiment it was defibrinated by stirring or
shaking with beads. The fibrin was removed by filtering through
gauze. Approximately one-half of the blood drawn was divided
into three samples and equilibrated for 25 minutes at 38°C. with
previously prepared gas mixtures containing about 20, 40, and
60 mm. of carbon dioxide. The other half was centrifuged to
obtain "separated" serum, separate portions of which were simi-
larly equilibrated with gas mixtures of the same composition as
used with whole blood.

After equilibration of the whole blood, the samples were trans-
ferred to ordinary centrifuge and hematocrit tubes containing
paraffin oil. The transfer was accomplished without contact
with any other gas. On the completion of centrifugation the
corpuscle volume was determined, and from the larger tubes serum
was obtained for the chloride and carbon dioxide analyses. All

possible precautions were taken to avoid or minimize loss of CO_2 from samples of blood and serum from the completion of equilibration to the transfer to the apparatus for CO_2 determination. After equilibration of the "separated" serum, the samples were transferred under oil to tubes suitable for withdrawing 1 cc. portions for carbon dioxide analyses.

Carbon dioxide analyses were carried out by transferring 1 cc. of serum directly from the tubes to the cup of the Van Slyke macro apparatus (1917). The calculations were made according to the details suggested by Van Slyke and Stadie (1921). Chloride determinations were made on picric acid filtrates using the volumetric principles of the McLean-Van Slyke (1915) titration. In our hands the end-point appeared a bit sharper when the buffer and starch were used in separate solutions. In this case the starch solution was prepared fresh every few days. We also found that picric acid filtrates gave a sharper end-point under any conditions than the trichloroacetic acid filtrates used in our previous work.

After equilibration was completed, the gas mixtures in our saturators were analyzed with a Haldane burette. Phosphate analyses were made by the colorimetric method described by Bell and Doisy (1920).

Correction for Change in Corpuscle Volume.

The increase of corpuscular volume at the expense of water from the serum, which results from absorption of CO_2 causes an increase in the concentration of all substances in serum and the amount of this increase must be deducted before calculating loss of chloride and gain of bicarbonate caused by increasing CO_2 tension.

In order that we might plot our values of chloride and bicarbonate and thereby secure the advantages of the graphic method, we have modified the method of correction for change in the volume of the corpuscles. In our first paper, the correction was applied by calculating the concentration of chloride and bicarbonate that would exist due to the change in volume of corpuscles *if no migration of ions had occurred*. For example, in Experiment 1, we have the data given in Table I.

As corrected formerly, the values were obtained in the following way:

$$\frac{56.2}{55.6} \times 0.1122 = 0.1134 \text{ M NaCl} \qquad \frac{56.2}{55.6} \times 0.0164 = 0.0166 \text{ M NaHCO}_3$$

This method is undesirable in that it leaves different basic values with which to compare each value obtained at higher tensions of CO_2, and such results for three tensions cannot be plotted on the same curve. It is preferable to correct all observed concentrations to *one basic volume* taking for this purpose the volume of the serum at the lowest carbon dioxide tension. The values for bicarbonate and chloride at the higher CO_2 tensions are merely multiplied by the necessary factor to give the respective concentrations that would exist in the serum had its volume not changed due to the taking up of water by the corpuscles.

TABLE I.

Experiment 1.

CO_2, *mm*	17.6	39.3	61.6
Serum, per cent of total blood volume	56.2	55.6	55.0
$NaHCO_3$, molar concentration	0.0164	0.0219	0.0257
NaCl, molar concentration	0.1122	0.1097	0.1085

TABLE II.

Correction for Change of Corpuscle Volume.

	Former method.		New method.	
CO_2, *mm*	17.6 to 39.3	17.6 to 61.6	17.6 to 39.3	17.6 to 61.6
NaCl-loss, *mols*	0.0037	0.0062	0.0036	0.0060
$NaHCO_3$-gain, *mols*	0.0053	0.0090	0.0053	0.0088
Percentage to loss of chloride	70	69	68	68

From the data just given, the correction by this method is:

$$\frac{55.6}{56.2} \times 0.1097 = 0.1086 \text{ M NaCl} \qquad \frac{55.6}{56.2} \times 0.0219 = 0.0217 \text{ M } NaHCO_3$$

The values for the highest tension of CO_2 are calculated in the same way using in this case the factor $\frac{55.0}{56.2}$ and the concentrations found by analysis. As shown by comparison in Table II, the numerical values obtained by the two methods are substantially the same. The new procedure has the advantage that the results may be plotted on a single graph.

The experimental data from the different samples of blood are given in detail in the protocols. The values for serum bicarbonate and chloride are corrected for change in serum volume as above described, and are brought together in Table III. In order to

TABLE III.

Loaned Buffer; Loss of Chloride and Gain of Bicarbonate. *

	CO_2	pH	$NaHCO_3$	Difference in $NaHCO_3$.	NaCl	Difference in NaCl.
	mm.		*mols*	*mols*	*mols*	*mols*
Dog 1.	17.6	7.57	0.0164		0.1122	
	39.3	7.34	0.0217	0.0053	0.1086	0.0036
	61.6	7.22	0.0252	0.0088	0.1062	0.0060
Dog 2.	18.5	7.51	0.0152		0.1122	
	42.5	7.30	0.0212	0.0060	0.1079	0.0043
	66.0	7.18	0.0244	0.0092	0.1042	0.0080
Dog 3.	18.5	7.53	0.0159		0.1110	
	37.3	7.34	0.0204	0.0045	0.1076	0.0034
	74.8	7.12	0.0243	0.0084	0.1042	0.0068
Dog 4.	20.0	7.50	0.0159		0.1182	
	44.3	7.27	0.0207	0.0048	0.1142	0.0040
	76.0	7.11	0.0237	0.0078	0.1091	0.0091
Man 1.	23.3	7.47	0.0175		0.1110	
	43.0	7.33	0.0230	0.0055	0.1071	0.0039
	72.5	7.17	0.0267	0.0092	0.1043	0.0067
Man 2.	16.7	7.65	0.0189		0.1103	
	35.2	7.44	0.0241	0.0052	0.1065	0.0038
	63.8	7.27	0.0290	0.0101	0.1020	0.0083

* Bicarbonate and chloride values have been corrected for change in corpuscular volume.

compare the relative participation of the various buffer factors, it is necessary to choose an arbitrary pH range, and to calculate the effect of each factor within this range. Without change in hydrogen ion concentration, the effect of the self-possessed serum buffers is, of course, zero, the buffer action being limited to that depending upon migration of acid to and from the cells. We have chosen the range of pH 7.45 to 7.25 as the basis of our calculations,

and in doing so it may be noted that we undoubtedly exaggerate the relative participation of the self-possessed buffers, which as pointed out by Van Slyke (1921) play less and less of a rôle under physiological conditions the smaller the difference in pH between arterial and venous blood. This range was chosen to encompass the varying values which appear to occur naturally, if not physiologically, and especially in order to minimize the errors in the determinations.

Since some of our experimental data commonly fall without this pH range, it is necessary to plot them in the form of curves and
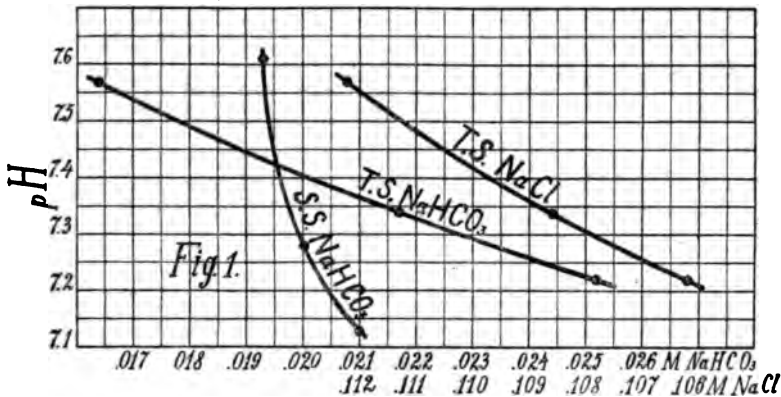
FIG. 1. Data are taken from Experiment 1 which was conducted on dog's blood. Chloride and bicarbonate values of true sera corrected for the change in volume of the corpuscles are plotted against pH.

obtain the desired values by interpolation. The most convenient method of treatment is to plot the molar concentrations of bicarbonate and of chloride of the true sera (corrected for change in serum volume) and the molar concentrations of bicarbonate of the separated serum against the pH which is calculated from the ratio of free and combined CO_2. The corrected data, given in Table III are plotted[2] in this way in Figs. 1, 2, and 3. From the

[2] Only three of the six charts are presented. The concavity to the abscissæ is greatest in Fig. 2, while the convexity is greatest in Fig. 3. All the curves of the other experiments occupy intermediate positions. It should be noted that if the true serum bicarbonate curve is convex to the abscissæ, the chloride curve varies from a straight line in the same direction. Generally this has been true in all our experiments. At present, we do not understand the significance of these variations.
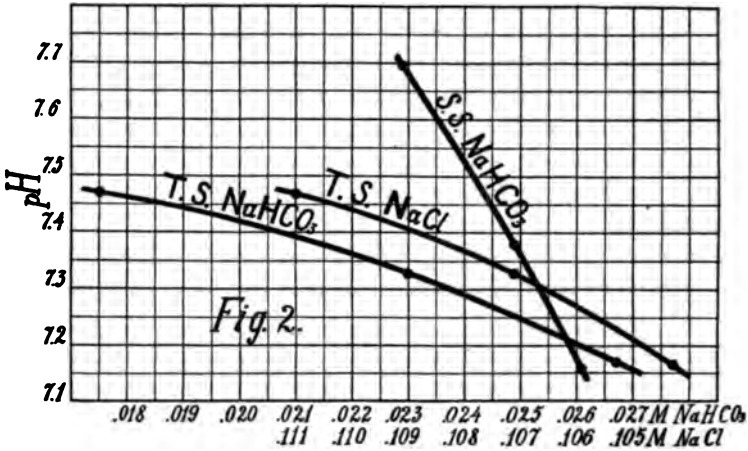
FIG. 2. Data are taken from Experiment 5 which was conducted on human blood. Chloride and bicarbonate values of true sera corrected for the change in volume of the corpuscles are plotted against pH.
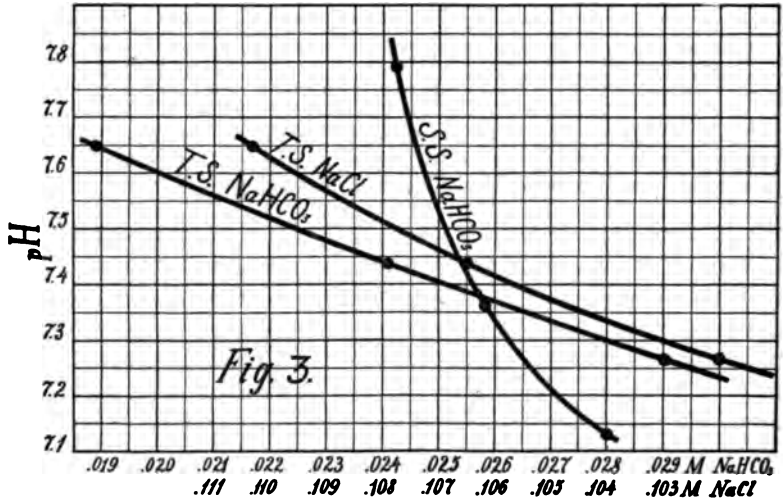
FIG. 3. Data are taken from Experiment 6 which was conducted on reduced human blood. Chloride and bicarbonate values of true sera corrected for the change in volume of the corpuscles are plotted against pH.

TABLE IV.

Serum Buffers, pH 7.45 to 7.25.

pH	True serum. Molar NaHCO₃.			Separated serum. Molar NaHCO₃.			True serum. Molar NaCl.			Percentage of base supplied from:		
	7.45	7.25	Gain.	7.45	7.25	Gain.	7.45	7.25	Loss.	Self-possessed buffers.	HCl migration.	Unaccounted for.
Dog 1......	0.0188	0.0242	0.0054	0.0195	0.0201	0.0006	0.1105	0.1069	0.0036	11	67	22
" 2......	0.0169	0.0225	0.0056	0.0163	0.0168	0.0005	0.1112	0.1066	0.0046	9	82	9
" 3......	0.0181	0.0222	0.0041	0.0154	0.0161	0.0007	0.1095	0.1061	0.0034	17	83	0
" 4......	0.0169	0.0211	0.0042	0.0225	0.0233	0.0008	0.1175	0.1138	0.0037	19	88	-7
Man 1......	0.0187	0.0250	0.0063	0.0245	0.0257	0.0012	0.1103	0.1056	0.0046	19	75	6
" 2......	0.0238	0.0296	0.0058	0.0253	0.0266	0.0013	0.1067	0.1016	0.0051	23	88	-11
Mean......										16	80.5	3+

curves in these figures, we read off the changes in concentration corresponding to the change in pH from 7.45 to 7.25 and these values are given in Table IV. Taking the data from Experiment 1 as an example, it will be seen (Table III) that on passing from a pH of 7.45 to 7.25, the true serum gained 0.0054 mols of bicarbonate, of which only 0.0006 mols or 11 per cent is due to the buffers of the separated serum (the self-possessed buffers), the remaining 0.0048 mols or 89 per cent being due to the presence of corpuscles (the loaned buffers). Of the latter, the disappearance of NaCl (by migration of HCl into the cells), 0.0036 mols, accounts for three-fourths, or for 67 per cent of the total gain. The remaining 22 per cent of the total gain (0.0012 mols) which is not accounted for in this experiment is perhaps due to the migration of other acids from serum salts into the cells. All of the other experiments show somewhat smaller fractions to be unaccounted for (from + 0.0005 to −0.0006 molar). The difficulty in the accurate measurement of small differences between much larger quantities is such that the variations between extremes is no greater than might be expected. The effect of small errors in individual values is considerable, as may be illustrated by the following calculations on the serum of Dog 1, Experiment 1.

Assuming that the basic value 0.1122 M NaCl is correct, let us see what effect an error of −0.0005 M or 0.5 per cent produces in our estimation of the loaned buffer value. The corrected value at 39.3 mm. of CO_2 $(0.1097 - 0.0005 = 0.1092.\ \ 0.1092 \times \dfrac{55.6}{56.2}$ $= 0.1080)$ becomes 0.1080 instead of 0.1086 and the loss of chloride is now 0.0042 instead of 0.0036. This accounts for $\left(\dfrac{0.0042 \times 100}{0.0053} = \right)$ 79 per cent of the total gain of bicarbonate instead of the 68 per cent actually found. Viewed in another way, an error of 0.5 per cent in the determination of one value produces a difference of about 12 per cent in the result. It is thus apparent that too much must not be expected from individual determinations. We, therefore, deem it more desirable to consider only the mean values. According to the average of all of our data, the buffer value of serum in the pH range of 7.45 to 7.25 is loaned to it to the extent of 80+ per cent by a migration of hydrochloric acid into the corpuscles; while the effect of the non-migrating self-

possessed buffers amounts to 16 per cent. This leaves 3 per cent unaccounted for and this is probably due to a migration of acids other than hydrochloric. The evidence presented by de Boer (1917) on the migration of SO_4^{--}, our results concerning phosphates, the behavior of oxalated blood already referred to, and particularly the fact that a large migration of H_2CO_3 into the corpuscles takes place, would seem to indicate that such a conclusion is justified.

We can feel fairly certain that we know the nature of all of the buffer reactions of the serum when the state of oxidation of the hemoglobin remains unchanged. In a recent paper, L. J. Henderson and coworkers (1919–20) state that when the oxygenated

TABLE V.

Alkali Furnished by Phosphate Buffer System.

Experiment.	Total gain of NaHCO₃ in separated sera. pH 7.45 to 7.25.	Total inorganic P of sera.	Base yielded by Na₂HPO₄ pH 7.45 to 7.25.	Percentage of total alkali supplied by Na₂HPO₄.	
				Separated sera.	True sera.
	molar	*molar*	*molar*	*per cent*	*per cent*
Dog 1.	0.0006	0.00129	0.0001	17	1.9
" 2.	0.0005	0.00174	0.00014	28	2.5
" 3.	0.0007	0.00084	0.00007	10	1.7
" 4.	0.0008	0.00119	0.0001	12	2.4
Man 1.	0.0012	0.00087	0.00007	6	1.1
" 2.	0.0013	0.0010	0.00008	6	1.4
Mean..................................				13	1.8

blood is reduced isohydrically about 60 per cent of the increased capacity of serum to bind CO_2, is due to a migration of chloride. Although this might seem to indicate some other loaned buffer mechanism, we shall attempt to show in a later paper that at least 90 per cent of the increased capacity is actually due to a shift of HCl. The lower estimate is due to neglecting the change in concentrations produced by the swelling of the corpuscles.

By means of our inorganic phosphate determinations we may calculate the participation of the serum phosphate in the self-possessed buffer action. By using the Hasselbalch equation,

$$pH = pK_1 + \log \frac{[Na_2HPO_4]}{[NaH_2PO_4]}$$

we can solve for the ratio of disodium to dihydrogen phosphate at pH 7.45 and 7.25 and from our data on serum inorganic phosphate calculate how much of the separated serum buffer value is due to the phosphate system. The fraction which this represents of the total base supplied by all buffers of separated serum shows the relative participation of the phosphates.

The data on this point are given in Table V. According to these calculations from 6 to 28 per cent of the self-possessed buffer action may be due to the phosphates; the remainder being due to salts of proteins and amino- and other organic acids. The serum phosphates appear to supply only from 1 to 3 per cent of the base contributed by the *total* serum buffers.

SUMMARY.

A series of experiments on samples of blood of four dogs and two men show that between the pH range af 7.45 and 7.25, the base furnished for the increase of bicarbonate in serum comes from the sources indicated (mean results):

(*a*) "Self-possessed", non-migrating serum buffers: 16 per cent. Of this, phosphates supply 1 to 3 per cent.

(*b*) "Loaned" buffer, due to presence of corpuscles: 84 per cent. Of this, migration of HCl into corpuscles liberates 80 per cent. The remaining 3 per cent is probably liberated from the salts of other acids, by migration of the latter into the corpuscles.

Protocols.

The blood was defibrinated by one of the usual procedures. Each gas-equilibrating mixture consisted of air plus CO_2 (except Experiment 6). With the exception of Experiment 4, no hemolysis occurred, and in this case it was very faint.

Experiment 1—Dog 1.

	True serum.			Separated serum.		
CO_2, *mm*	17.6	39.3	61.6	18.8	41.8	62.4
Total CO_2, *vols. per cent*	37.9	51.9	62.0	44.5	47.9	51.6
Dissolved CO_2, *vols. per cent*	1.25	2.80	4.38	1.34	2.98	4.44
$BHCO_3$, *vols. per cent*	36.6	49.1	57.6	43.2	44.9	47.2
pH	7.57	7.34	7.22	7.61	7.28	7.13
NaCl, *mols*	0.1122	0.1097	0.1085			
Serum, *per cent*	56.2	55.6	55.0			
Inorganic P, *mg*				4.0		

Experiment 2—Dog 2.

	True serum.			Separated serum.		
CO$_2$, *mm*	18.5	42.5	66.0	19.7	39.8	65.0
Total CO$_2$, *vols. per cent*	35.4	51.4	61.1	37.7	40.8	44.8
Dissolved CO$_2$, *vols. per cent*	1.32	3.03	4.70	1.40	2.83	4.63
BHCO$_3$, *vols. per cent*	34.1	48.4	56.4	36.3	38.0	40.2
pH	7.51	7.30	7.18	7.51	7.23	7.04
NaCl, *mols*	0.1122	0.1099	0.1078			
Serum, *per cent*	48.2	47.3	46.6			
Inorganic P, *mg*				5.4		

Experiment 3—Dog 3.

	· True serum.			Separated serum.		
CO$_2$, *mm*	18.5	37.3	74.8	18.0	41.2	71.6
Total CO$_2$, *vols. per cent*	37.0	48.8	61.3	35.0	39.5	43.1
Dissolved CO$_2$, *vols. per cent*	1.32	2.66	5.32	1.28	2.93	5.10
BHCO$_3$, *vols. per cent*	35.7	46.1	56.0	33.7	36.6	38.0
pH	7.53	7.34	7.12	7.52	7.20	6.97
NaCl, *mols*	0.1110	0.1088	0.1073			
Serum, *per cent*	56.2	55.6	54.6			
Inorganic P, *mg*				2.6		

Experiment 4—Dog 4.

	· True serum.			Separated serum.		
CO$_2$, *mm*	20.0	44.3	76.0	16.1	41.6	67.6
Total CO$_2$, *vols. per cent*	37.1	50.3	61.0	50.5	54.2	58.6
Dissolved CO$_2$, *vols. per cent*	1.42	3.15	5.41	1.15	2.96	4.81
BHCO$_3$, *vols. per cent*	35.7	47.1	55.6	49.3	51.2	53.8
pH	7.50	7.27	7.11	7.73	7.34	7.15
NaCl, *mols*	0.1182	0.1161	0.1140			
Serum, *per cent*	56.7	55.8	54.3*			
Inorganic P, *mg*				3.7		

* Probably erroneous; change larger than expected.

Experiment 5—Man 1.

	True serum.			Separated serum.		
CO_2, *mm*..................	23.3	43.0	72.5	18.1	41.2	71.2
Total CO_2, *vols. per cent*......	40.9	55.4	66.4	52.6	58.7	63.5
Dissolved CO_2, *vols. per cent*..	1.66	3.06	5.16	1.29	2.93	5.07
$BHCO_3$, *vols. per cent*........	39.2	52.3	61.2	51.3	55.8	58.4
pH..........................	7.47	7.33	7.17	7.70	7.38	7.16
NaCl, *mols*..................	0.1110	0.1087	0.1065			
Serum, *per cent*...............	54.1	53.3	53.0			
Inorganic P, *mg*..............				2.7		

Experiment 6—Man 2.

	True serum.*			Separated serum.		
CO_2, *mm*..................	16.7	35.2	63.8	15.6	44.7	82.0
Total CO_2, *vols. per cent*.......	43.5	57.1	71.3	55.3	61.0	68.6
Dissolved CO_2, *vols. per cent*..	1.19	2.50	4.54	1.11	3.18	5.83
$BHCO_3$, *vols. per cent*........	42.3	54.6	66.8	54.2	57.8	62.8
pH..........................	7.65	7.44	7.27	7.79	7.36	7.13
NaCl, *mols*..................	0.1103	0.1076	0.1047			
Serum, *per cent*...............	56.5	55.9	55.0			
Inorganic P, *mg*..............				3.1		

* The true serum of this blood was obtained by equilibration with H_2 + CO_2. Approximately 95 per cent of the hemoglobin was in the reduced form.

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STUDIES ON ENZYME ACTION.

XX. THE PROTEASE ACTIONS OF MALIGNANT HUMAN AND RAT TUMOR EXTRACTS AT DIFFERENT HYDROGEN ION CONCENTRATIONS AND IN THE PRESENCE OF VARIOUS SALTS.

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INTRODUCTION.

The chemical changes which occur in living matter are profoundly influenced, and even may be said to be controlled, by enzymes and their actions.[1] Thus, different enzymes in different parts of a living organism may produce different products from one and the same mixture supplied to the different parts. Strictly speaking, this statement should be reversed. Since different products are obtained in different parts of a living animal, for example, from the circulating blood, the presence of various enzymes in these different parts is inferred, since enzymes, at present, are known only by their actions. Without entering farther into these questions in the present connection, the importance of enzyme actions in the chemical changes of living matter is definitely recognized. As a logical development, a modification of such enzyme actions might well be expected in pathological conditions. This investigation was therefore begun to study certain enzymes which may be involved in malignant growths. The results in this paper are limited to the study of the proteolytic enzyme, and refer to the actions obtained with various human malignant tumors as well as the Flexner-Jobling rat carcinoma. The effect on the protease actions of different hydrogen ion con-

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[1] *Cf.* for example Falk, K. G., Catalytic action, Chapter VII, A chemical interpretation of life processes, New York, 1922.

centrations, the determination of the optimum hydrogen ion concentration, the change in action with time and with concentration of substrate and of enzyme material, and the effect of a number of neutral salts on the action at the optimum hydrogen ion concentration, were studied.

Experimental Methods.

Preparation of Materials for Enzyme Tests.—The solutions and mixtures used for the enzyme experiments were obtained from human tumors and rat tumors. With the former, the material was prepared from specimens removed in surgical operations, within 2 to 3 hours after the operations. With the latter, the neoplasm was removed from the animal after killing it with ether according to the method described in detail by Sugiura and Benedict.[2] In both cases, after the removal of all non-neoplastic material, including fibrous tissue, fat, muscle, etc., the tumor was cut into small pieces, washed with physiological salt solution, and dried by means of filter paper. A small portion of the tumor was dried in an air oven at 102° in order to determine the water content. The remainder was weighed, macerated with sea sand, treated with a definite quantity of water or saline solution, shaken thoroughly, and after the addition of a small quantity of toluene, placed in an ice box over night. The mixture was then centrifuged and filtered, either through paper or with suction through a Gooch crucible containing a thin mat of long fibred asbestos.

The tumors of human origin were obtained at more or less irregular intervals as the surgical material presented itself through the courtesy of Dr. E. T. Leddy of the Staff of Memorial Hospital. The characters of the growths as given in Table I were obtained from the records of the Pathological Department of the Hospital. The rat tumors were obtained from albino rats, both male and female, which were inoculated with the Flexner-Jobling rat carcinoma in the usual way and allowed to grow for a period of from 3 to 6 weeks.[2] The term $\frac{FRC,}{86A}$ for example, indicates that the tumor used was derived from the 86th generation, Series A. The rats were fed on wheat bread soaked in whole milk, fresh cabbage or carrots, and fresh tap water *ad libitum.*

[2] Sugiura, K., and Benedict, S. R., *J. Cancer Research*, 1920, v, 373.

Protease Methods.—The proteolytic actions of the tumor extracts were studied by the formol and the Van Slyke amino nitrogen methods on casein and on a peptone preparation.[3] Not all extracts were tested by both methods with both substrates, but enough experiments were carried out to make definite conclusions possible.

For the Van Slyke amino nitrogen method the micro apparatus was used and the customary precautions taken.[4] The use of casein as substrate caused a certain amount of trouble. In solutions more acid than pH 7.0, the presence of casein in suspension necessitated some care in order to run the requisite quantity of mixture into the reaction bulb, while the introduction of the casein into the acid mixture in the reaction bulb in every case resulted in the casein flocculating out. In order to have comparable conditions and also to cause the reaction to proceed to completion as far as practicable, the reaction bulb was shaken for 8 minutes in every determination. Either 2 or 3 cc. were used in the determinations.

In carrying out the determinations by the Sörensen formol method, 15 to 25 cc. of the mixtures and 0.1 N sodium hydroxide solution were used. Phenolphthalein was used as indicator. The titrations were divided into two steps, first to a definite pink color (direct), then after the addition of 10 to 15 cc. of 35 per cent formaldehyde solution neutralized toward phenolphthalein, to a definite pink color again (formol). The results presented in this paper are comparative; that is to say, the enzyme actions were obtained by subtracting from the results with enzyme-substrate mixtures, the sums of the separate results of the enzyme-water (or solution) and substrate-water (or solution).

Preparation of Solutions.—The pH values of the various solutions were determined by means of indicators and the standard solutions recommended by Clark.[5] The latter were checked up potentiometrically. A number of the peptone solutions were tested potentiometrically and as a result of these tests the indicators were chosen which gave the most satisfactory color comparisons. The substrate solutions in the earlier experiments were

[3] Obtained from Fairchild Bros. and Foster, New York.

[4] Van Slyke, D. D., *J. Biol. Chem.*, 1913–14, xvi, 121.

[5] Clark, W. M.. The determination of hydrogen ions, Baltimore, 1920.

prepared by direct weighings of the different peptone and casein samples.   Later, a stock solution was prepared for any one series and   hydrogen   ion   concentration,   and   suitable   portions   were pipetted from this.

Casein was used as substrate as a type of complex protein, while the peptone was used because of its more ready handling under the given conditions.   The results and conclusions with these two substrates were essentially the same.

In all the experiments except those involving neutral salt actions, 0.1 N sodium hydroxide or hydrochloric acid was used to bring the solutions for the enzyme tests to the required hydrogen ion concentrations.   In the neutral salt tests, the hydroxyl or hydrogen compound of the metallic or acid component of the neutral salt was used in order to avoid the introduction of a different salt into the mixture.

Toluene was present in every mixture throughout the tests.

EXPERIMENTAL RESULTS.

Protease Actions.

General Data.—The data relative to the natures, amounts, and treatments of the tumors for the purpose of obtaining extracts for the protease tests are given in Table I.

The amounts to which the various extracts, dialyzed and not dialyzed, were diluted in the different protease tests are not given directly in the table.   The final results of such dilutions are shown, however, in the last column, in which are given per cc. of the mixtures tested the quantities of original tumors extracted.

The extracts in the first eleven experiments, in Experiments 30, 31, and 32, and in part of Experiment 28 were used directly. The other extracts were dialyzed over night in collodion bags against running tap water.   The pH of this tap water was 7.0 to 7.2, and its temperature varied between 10 and 20° at the different times.   The volumes of the solutions increased between 20 and 50 per cent in the dialyses.   These increases were taken into account in calculating the final concentrations.

The filtered rat tumor extracts were colorless to very light yellow.   Some of the human tumor extracts were red in color

(because of blood), but on dilution in preparing the mixtures for the tests the color was found not to interfere. The pH values of the various extracts varied between 6.6 and 7.0. With some of the extracts the addition of acid to bring them to the required pH value produced a faint turbidity or cloudiness.

The special treatments and explanations with reference to the protease actions will be given in connection with the separate results. Unless stated differently, the reactions were allowed to proceed for 22 hours at 38° and the actions then determined.

The presentation of the results of the protease actions presents certain difficulties. The concentration of the substrate can be kept uniform in the various series of experiments, but every tumor extract is likely to be different even if the concentrations of the solid tumors as extracted had been kept contant. Each series with any one tumor preparation should, strictly speaking, therefore, be considered separately. The presentation of the individual experiments is, however, impracticable, in view of their large number. The results will therefore be given in the first instance in terms of a common unit of volume and concentration of substrate, that is, as amounts of protease action per cc. of mixture tested; and in the second instance as relative actions by considering the maximum action observed in any one series as 100 per cent and calculating the other actions of this series in terms of this. In the second way, the differences in the enzyme preparations are eliminated, and the relative actions studied on a common basis.

The Van Slyke method gives directly the increase in the concentration of amino groups formed on proteolysis, after proper corrections for blanks have been made. Nothing is indicated as to other reactions which may have occurred.

The formol method gives the concentration of acid produced in the reaction. This acid may be formed as a result of the hydrolysis of a peptide linking, and also of any other decomposition or hydrolysis not necessarily accompanied by the simultaneous formation of an amino group as in the peptide hydrolysis. Also, the formol titration may indicate the production of ammonia, present as an ammonium salt before the addition of formaldehyde, the acid component of the ammonium salt being determined in the formol titration. The change in hydrogen ion concentration of the mixture would show any pronounced acid or alkali formation

TABLE I.

Data Relative to the Treatment of Tumors for Protease Experiments.

Experiment No.	Source of tumor.	Character of tumor.	Fresh tumor.	Water content of tumor.	Extracting solution. Nature.	Amount.	Filtered tumor extract treatment.	Tumor content of solution in protease experiments.
			gm.	*per cent*		*cc.*		*mg. per cc.*
2	Human.	Epidermoid carcinoma of cervix.	2.0	82.2	0.9 per cent NaCl.	50		2.1
3a	Rat.	$\frac{FRC}{87A}$, active tissue.	2.66	85.2	0.9 "	50		3.1
3b	"	$\frac{FRC}{87A}$, necrotic tissue.	2.29	84.6	0.9 "	50		2.3
5a	Human.	Neurogenic sarcoma of thigh.	21.0	82.4	0.9 "	100		19.1
5b	"	Neurogenic sarcoma of thigh.	21.0	82.4	Water.	100		19.3
6	"	Papilloma of bladder.	5.1	81.6	"	100		4.1
7	"	Squamous carcinoma of antrum.	2.23	81.3	"	50		1.8
8	Rat.	$\frac{FRC}{87A}$	11.3	81.2	"	100	a. Unfiltered. b. Filtered through paper. c. Filtered through asbestos.	3.0

9	Rat.	$\dfrac{FRC}{87A}$		10.2	83.5	Water.	100		2.7
10	"	$\dfrac{FRC}{87A}$ and $\dfrac{FRC}{87B}$		30.1	83.0	"	100		24.1
11	Human.	Neurosarcoma of back.			87.5	"	100		31.5
12	Rat.	$\dfrac{FRC}{89B}$		45.1	84.9	"	150	Dialyzed.	69.4
13	Human.	Papillary adenocarcinoma of parotid gland.		68.3	78.3	"	100	"	98.8
14	Rat.	$\dfrac{FRC}{89B}$		26.9	85.1	"	100	"	29.8
15	"	$\dfrac{FRC}{89B}$		15.7	83.5	"	100	"	46.5
16	"	Spontaneous tumor; spindle-celled sarcoma.		12.3	86.0	"	100	"	37.5
17	Human.	Neurosarcoma of arm.		13.3	86.5	"	100	"	17.2
18	Rat.	$\dfrac{FRC}{90A}$		22.6	83.8	"	100	"	58.6

TABLE I—*Concluded*

Experiment No.	Source of tumor.	Character of tumor.	Fresh tumor.	Water content of tumor.	Extracting solution. Nature.	Extracting solution. Amount.	Filtered tumor extract treatment.	Tumor content of solution in protease experiments.
			gm.	*per cent*		*cc.*		*mg. per cc.*
19	Human.	Endothelioma of sarcum.	93.4	81.9	Water.	250	Dialyzed.	42.5, 85.0 170.0, 102
20	Rat.	$\frac{FRC}{90C}$	28.4	85.6	"	100	"	32.1 to 128.4
21	Human.	Myxosarcoma of leg.	39.8	87.2	"	100	"	129.0
22	"	Gliomyoma of uterus.	150.0	79.0	"	300	"	156.3 and 195.4
23	Rat.	$\frac{FRC}{91A}$	41.5	83.6	"	150	"	114.6
24	"	Normal muscle tissue of hind legs.	56.5	78.0	"	200	"	121.3
25	"	Normal muscle tissue of hind legs.	61.9	75.9	"	200	"	126.9
26	"	$\frac{FRC}{91C}$	51.0	82.9	"	150	"	110.7
27	"	$\frac{FRC}{96A}$	30.0	84.2	"	100	"	83.7

28	Rat.	$\frac{FRC}{96B}$	30.3	85.0	Water.	100	a. Dialyzed. b. Not dialyzed.	61.2 61.2
29	"	$\frac{FRC}{96B}$	12.9	83.7	"	50	Dialyzed.	60.4
30	Human.	Melanoma of femoral triangle.	148.0	79.6	"	300	Dialyzed; not dialyzed.	98.7
31	Rat.	$\frac{FRC}{96C}$	29.0	85.5	"	100		55.1
32	Human.	Papillary adenocarcinoma of cervix.	34.5	82.6	"	100		69.0
34	Rat.	$\frac{FRC}{98B}$	33.8	84.1	"	150	Dialyzed.	60.7
35	"	$\frac{FRC}{98B}$	36.9	84.7	"	150	"	80.8
36	"	$\frac{FRC}{99A}$	25.2	84.0	"	100	"	61.9
37	"	Normal muscle tissue of hind leg.	54.4	84.7	"	150	"	111.6
39	Human.	Papillary adenocarcinoma of ovary.	83.7	83.9	"	200	"	137.4

in the reaction. The formol method evidently, therefore, does not yield results theoretically as simple and as clear-cut as the Van Slyke method.

In the experiments in which the mixtures at the beginning of the actions were treated with acid or alkali so that their pH values varied from 4.0 to 9.0, the direct titrations brought all the mixtures to the same pH (very nearly 8.5), and the formol titrations then gave the carboxyl groups produced in the proteolytic actions. These formol titrations would be expected to give smaller values than would be obtained in the corresponding Van Slyke amino nitrogen determinations for the hydrolysis of the peptide linkings corresponding to the proteolyses. Each series would be complete in itself, but a more or less constant factor would represent the differences between the two. If the formol titration is started at pH 7.0, however, the Van Slyke and formol methods give essentially the same results as shown in the study of the hydrolysis of gelatin by Northrop,[6] and in the results which will be communicated in this paper.

In order to have as great a uniformity as possible, the results will be given in terms of milligrams of nitrogen in the form of amino groups produced by the protease actions in 100 cc. of the mixtures at the temperature 38° and in the time indicated, corrected for enzyme and substrate blanks. They are obtained directly from the determinations by the Van Slyke method. In the formol method, the hydrolysis of the peptide linking is assumed to be the only reaction occurring. In the series in which the actions at different hydrogen ion concentrations were studied, the results for the direct titrations will be indicated as milliequivalents of acid (or alkali for the negative acid values) per 100 cc. of mixture, and for the formol titrations as milligrams of nitrogen per 100 cc. Both titrations were carried out with phenolphthalein as indicator. The formol titration results are not the same numerically as the Van Slyke results, but the comparative changes and the optima observed are the same. In the other series, the formol method was carried out with the solutions initially at pH 7.0. Although the titrations were carried out in two steps, direct and formol, with phenolphthalein as indicator in each, the results will be combined and presented as one titration

[6] Northrop, J. H., *J. Gen. Physiol.*, 1920–21, iii, 715.

in terms of milligrams of amino nitrogen formed per 100 cc. of mixture.

Protease Actions at Different Hydrogen Ion Concentrations.—The first problem studied included the determination of protease activities at different hydrogen ion concentrations with a view to finding the optimum conditions for the actions. Preparations from Tumors 2 to 11 were used for this purpose. None of these extracts was dialyzed before testing. Table II shows some of the experimental results which were obtained. In all these experiments 1 cc. of the mixture contained 8 mg. of peptone or casein. In Table III and Fig. 1 are presented the results for all the experiments in which more or less complete series were obtained at a number of different hydrogen ion concentrations. A number of series were obtained with results for pH 4.0, 6.0, and 8.0, only. These confirm in general the more complete series and will not be given in detail.

The main conclusion to be drawn from these results is that a definite optimum for the actions is observable at pH 7.0, with a more gradual decrease toward pH 8.0 than toward pH 6.0.

A few explanations and further conclusions with reference to these experiments may be given. Extracts of Tumor 5 were prepared both with water and with physiological salt solution. These were tested at pH 4.0, 6.0, and 8.0, and gave practically identical results. All the subsequent extractions were therefore carried out with water only. Extracts of Tumors 2 and 3 were also tested only at pH 4.0, 6.0, and 8.0, and are not included in the results. The negative values of a number of the direct titrations, especially with peptone, are of interest. The hydrolysis evidently produced an excess of the alkaline products (probably ammonia) over the acid. The amounts in most cases, especially with the initial pH values of 5.0, 6.0, and 7.0, are extremely small, but unquestionably are correct. They do not, however, appear to effect the formol titrations. Experiment 7 gave unaccountably large negative direct titration values.

The extracts of Tumors 8 and 9 were tested for protease actions unfiltered, filtered through paper, and filtered through asbestos. No regular difference was observed to indicate that either method of filtration removed active material.

TABLE II

Protease Actions of Tumor Extracts at Different Hydrogen Ion Concentrations.

Experiment No.	Substrate.	Method.	pH 4.0	5.0	6.0	7.0	8.0	9.0
6	Peptone.	Direct, milli-equivalents acid per 100 cc........		0	−0.01	−0.10	'	−0.11
		Formol, mg. N per 100 cc........		1.3	3.7	4.1	4.1	0.9
7	"	Direct, milli-equivalents acid per 100 cc........		−0.16	−0.29	−0.18	−0.14	−0.34
		Formol, mg. N per 100 cc........		2.2	3.9	5.0	4.9	2.7
8a	"	Direct, milli-equivalents acid per 100 cc........		−0.01	−0.08	−0.14	−0.31	−0.16
		Formol, mg. N per 100 cc........		3.4	3.1	4.3	2.7	2.4
8b	"	Direct, milli-equivalents acid per 100 cc........		0.06	−0.02	0.04	0.15	−0.11
		Formol, mg. N per 100 cc........		2.0	4.1	5.3	3.9	1.5
8c	"	Direct, milli-equivalents acid per 100 cc........		0.02	−0.04	−0.06	−0.14	−0.31
		Formol, mg. N per 100 cc........		1.6	3.8	5.2		2.2
8a	Casein.	Direct, milli-equivalents acid per 100 cc........		0.01	0.12	−0.02	−0.06	0.01
		Formol, mg. N per 100 cc........		1.4	1.5	3.4	0.8	0.8
8b	"	Direct, milli-equivalents acid per 100 cc........		0.04	0.05	−0.04	−0.13	−0.12
		Formol, mg. N per 100 cc........		0.6	0.9	1.5	1.2	0.8
8c	"	Direct, milli-equivalents acid per 100 cc........		0.08	0.07	−0.03	−0.15	−0.09
		Formol, mg. N per 100 cc........		1.6	0.9	1.6	1.2	0.5
10	Peptone.	Van Slyke, mg. N per 100 cc........	1.8	4.4	8.0	12.2	11.2	6.4

TABLE III.

Percentage Protease Actions of Tumor Extracts at Different Hydrogen Ion Concentrations.

Experiment No.	Substrate.	Method.	pH					
			4.0	5.0	6.0	7.0	8.0	9.0
6	Peptone.	Formol.		32	90	100	100	22
7	"	"		44	78	100	98	54
8a	"	"		79	72	100	63	56
8b	"	"		38	77	100	74	28
8c	"	"		31	73	100		42
9a	"	"		50	85	100	85	21
9b	"	"		39	61	100	74	77
8a	Casein.	Formol.		42	44	100	24	24
8b	"	"		40	60	100	80	53
8c	"	"			56	100	75	31
9a	"	"		55	39	100	64	18
9b	"	"		59	71	100	53	0
9c	"	"			40	100	93	47
10	Peptone.	Van Slyke.	15	36	65	100	92	52
11	"	" "	16	45	50	100		
Averages.								
	Peptone.	Formol.		45	77	100	82	43
	Casein.	"		49	52	100	65	29
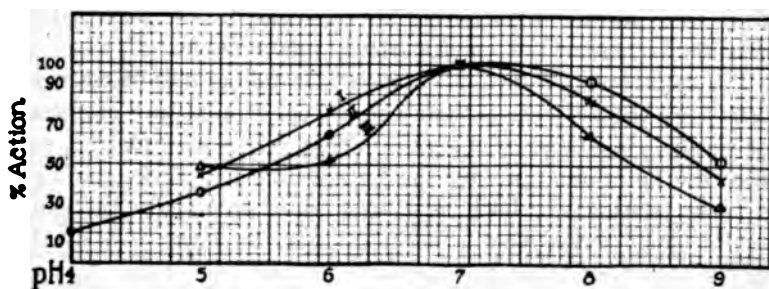
Fig. 1. Curves showing the relations between the protease actions of tumor extracts (percentages of the maximum actions plotted as ordinates and the corresponding pH values of the extracts as abscissæ).

Curve I represents actions on peptone, averages of the results obtained by the Sörensen formol method; Curve II, actions on peptone, results obtained by the Van Slyke amino nitrogen method; Curve III, actions on casein, averages of the results obtained by the Sörensen formol method.

Comparison between the experimentally observed values in the different experiments is not feasible because of the different concentrations of tumor materials in the various mixtures. However, of those quoted so far, all except Experiment 10 were of the same order of magnitude as far as concentration of extracted material was concerned, and gave actions also of the same order. Experiment 10 with tumor in greater concentration gave considerably greater actions.

The variation in protease action with the hydrogen ion concentration is brought out clearly in Table III (and Fig. 1) which also permits of a comparison of the different experiments from this point of view. The maximum action in any one series is placed at 100 and the actions at the other hydrogen ion concentrations calculated in terms of this. Although there are several minor discrepancies, the general relation of a distinct optimum at pH 7.0 is brought out clearly.

Rate of Protease Actions.—A number of series of experiments were carried out to determine the amounts of the actions after definite time intervals. Extracts from tumors, or Preparations 19, 22, 23, 24, and 25 were used. 1 cc. of mixture contained 20 mg. of peptone in every case, except Experiment 19 where 16 mg. were present per cc. Both the formol and Van Slyke methods were used. The tests were run at pH 7.0. The formol results include the sums of the direct (which were very small in every case) and formol titrations. In experiments of this nature occasional irregularities are apparently unavoidable.

The experimental results expressed as milligrams of nitrogen formed by the protease actions per 100 cc. of mixtures, corrected for blanks, are given in Table IV. These results were plotted, and since the determinations by the formol and Van Slyke methods in those cases where both were used gave practically the same results, such series were combined. Fig. 2 shows two of the curves obtained; those for Extracts 22 and 24. Table V then gives the values for the actions at more regular time intervals as taken from the various curves for the experimental results shown in Table IV.

Although it is difficult to compare enzyme actions from different materials in a quantitative manner, some conclusions may be

drawn relative to the kinetics of the reactions. The data presented in Table V will be used.

Extracts 19 and 22 were obtained from tumors of human origin, Extract 23 from a tumor of rat origin, Extracts 24 and 25 from normal rat tissue. Very nearly the same weights of tumor were extracted per cc. of final mixture.

Comparing the actions of the last three extracts (material from rats), it is seen that the tumor protease showed much greater activity than the protease of normal tissue. No special significance should be attached to this fact, however, since the cell

TABLE IV.

Experimental Results on the Rates of Protease Actions of Extracts.

Extract 19.		Extract 22.			Extract 23.			Extract 24.			Extract 25.		
Time.	Van Slyke method.	Time.	Van Slyke method.	Formol method.	Time.	Van Slyke method.	Formol method.	Time.	Van Slyke method.	Formol method.	Time.	Van Slyke method.	Formol method.
min.		min.			min.			min.			min.		
0	0	0	0	0	0	0	0	0	0	0	0	0	0
55	1.2	115	9.2	9.1	190	11.2	8.3	210	2.1	3.4	215	0.4	5.4
105	1.6	185	10.5	10.7	290	11.2	11.0	285	2.9	2.9	1,710	22.4	19.0
250	5.4	300	13.9	17.9	1,335	26.9	26.4	1,675	11.5	13.1	1,730	21.1	
370	5.4	1,295	25.4	23.8	1,435	26.5	28.0	2,895	19.9	16.6	2,790	20.9	22.7
1,675	14.6	1,395	22.7	23.0	1,735	29.0	29.1	3,135		17.4			
1,735	15.2	1,775		24.9	3,070	39.0	39.0						
3,135	19.1	2,845	29.1	29.3									

characters were entirely different. Except for Extract 25, none of the actions appeared to have reached a limit.

Attempts to apply methematical equations to the kinetic relationships of these protease actions are as inconclusive as with most enzyme actions. This may be illustrated by the application of Schütz's rule[7] to the results given in Table V. Schütz's rule states that the amounts of enzyme action are proportional to the square roots of the times of actions. Table VI gives the values for K as derived from the expression

[7] Schütz, E., *Z. physiol. Chem.*, 1885, ix, 577.
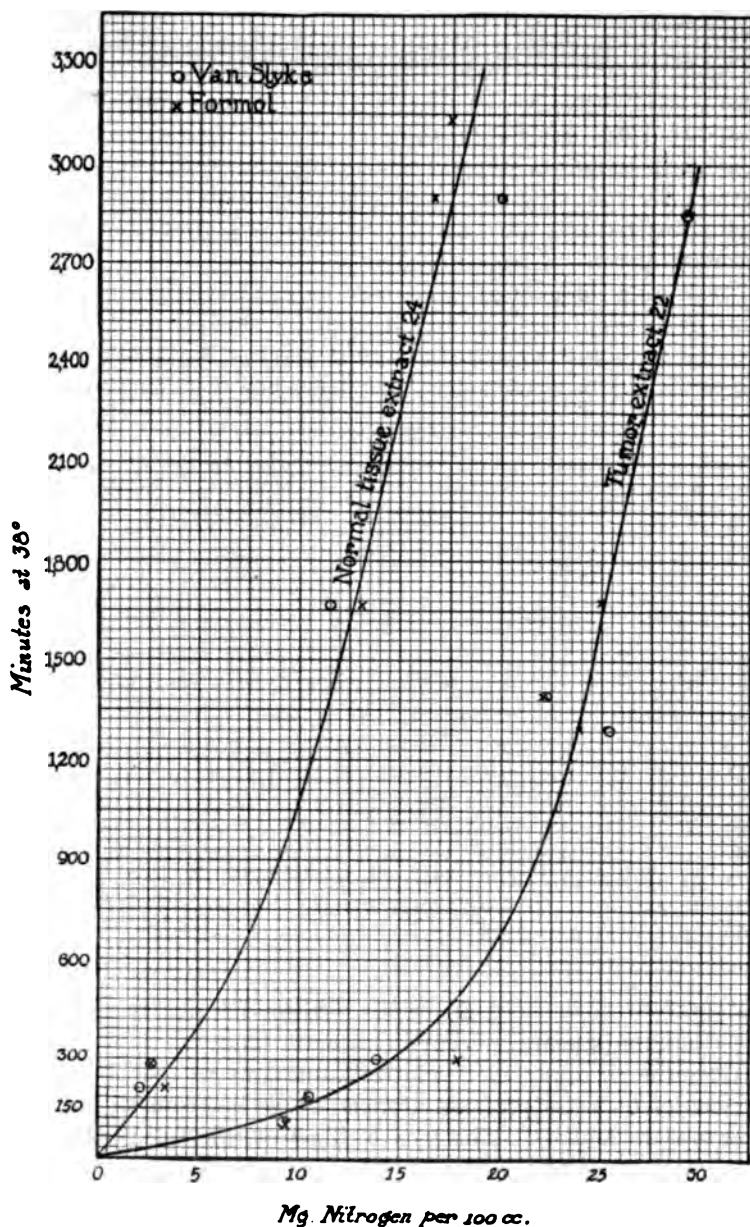
FIG. 2. Rates of protease actions of extracts on peptone at pH 7.0. Times of actions (minutes) plotted as ordinates; amounts of actions (as milligrams of amino nitrogen produced per 100 cc. of mixtures), as abscissæ.

Curve 22 represents results obtained with tumor extract 22; Curve 24, results with normal tissue extract 24.

$$K = \frac{x}{\sqrt{E\,T}}$$

in which x represents the change in time, T, for enzyme concentration, E. E is taken to be constant in each series.

TABLE V.

Rates of Protease Actions of Extracts Taken from Curves of Experimental Results.

Time.	Human.		Rat tumor.	Normal rat.	
	Extract 19.	Extract 22.	Extract 23.	Extract 24.	Extract 25.
min.					
0	0	0	0	0	0
60	1.0	5.2	3.0	0.9	1.6
120	1.9	8.5	5.5	1.7	3.1
240	3.7	12.8	9.6	3.2	5.9
480	6.7	17.7	15.2	5.8	10.3
720	9.2	20.3	19.5	7.8	13.6
960	11.2	22.0	22.7	9.3	16.2
1,200	12.7	23.2	25.5	10.6	18.1
1,440	14.0	24.2	27.7	11.7	19.5
1,920	15.8	26.0	31.6	13.7	21.3
2,400	17.3	27.6	34.8	15.6	22.0
2,880	18.5	29.3	37.9	17.5	22.1

TABLE VI.

Values of the Constant of Schütz's Rule Derived from the Results of Table V.

Time.	Extract 19.	Extract 22.	Extract 23.	Extract 24.	Extract 25.
min.					
60	0.13	0.67	0.39	0.12	0.21
120	0.17	0.78	0.50	0.16	0.28
240	0.24	0.83	0.62	0.21	0.38
480	0.31	0.81	0.69	0.27	0.47
720	0.34	0.76	0.73	0.29	0.51
960	0.36	0.71	0.73	0.30	0.52
1,200	0.36	0.66	0.72	0.30	0.51
1,440	0.37	0.64	0.73	0.31	0.51
1,920	0.36	0.59	0.72	0.31	0.49
2,400	0.35	0.56	0.71	0.32	0.45
2,880	0.35	0.55	0.71	0.33	0.41

It was shown by Northrop[6] in interpreting the mechanism of Schütz's rule, that the rule involves the assumptions that the concentrations of the products of reaction are large compared to the concentration of enzyme, and that the quantity of substrate remains fairly constant.   These conditions hold only after the reaction has proceeded to a certain extent, and during the time that a certain amount of action occurs.   In the reaction between pepsin and albumin, Northrop found that the constant calculated according to Schütz's rule first increased with the time, then remained constant for various lengths of time, and then decreased.   Exactly the same behavior is shown by the results given in Table VI for the extracts from the three sources.   All the actions showed initial increases, then constancy for different periods, and then more or less irregular decreases.   Northrop developed an equation to include such factors as varying enzyme concentration, which would be expected to hold for practically the whole course of the reaction.   The application of this equation, involves, however, the knowledge of the final value of the enzyme action (hydrolysis in this case).   The results obtained in this investigation do not permit of the determination of these final values, since there are, at least in four of the five series, no indications that the reactions had approached completion.   It might be thought that the consideration of this expression as one containing two unknowns, the constant and the final value of the actions, evaluating perhaps by the method of least squares, would give results of interest. This method of treatment, while employed at times, really assumes the fact which is being tested.

The results given in this section may perhaps be interpreted more satisfactorily when the mechanism of enzyme actions shall have been formulated more completely.   Until then, the results must be considered as empirical contributions, which agree with the observations of others as far as comparison at the present time is possible.

Relations between the Protease Actions and the Changes in Enzyme and Substrate Concentrations.—Two series of experiments were carried out with extracts of Tumors 19 and 20 (one of human and one of rat origin) in which the concentrations of enzyme material and of substrate were varied.   The relative enzyme concentra-

[6] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 471.

tions were in the ratios 1:2:4 in both series. With Tumor Extract 19, the peptone concentrations were in the ratios 1:2.5:5, ranging from 2.65 to 13.2 mg. of peptone per cc. of final mixture; with Tumor Extract 20, their concentrations were in the ratios 1:2.5:5:10; ranging from 2.0 to 20 mg. per cc. of final mixture. The experiments were carried out for 22 hours at 38°. The Van Slyke method was used for the determinations.

Table VII shows the results of the protease actions expressed as milligrams of amino nitrogen produced per 100 cc. of mixture.

TABLE VII.

Effect of Change in Concentration of Enzyme and Substrate on Protease Actions of Tumor Extracts.

	Concentration of peptone.			
	1.0	2.5	5.0	10.0
Concentration of tumor extract 19.				
1	4.4	6.6	7.7	
2	5.3	9.8	16.4	
4	9.3	14.6	24.2	
Concentration of tumor extract 20.				
1	3.9	5.8	11.1	22.3
2	4.8	11.1	16.5	25.1
4	5.7	14.8	20.8	39.5

In order to study the effects of changes in concentrations of enzyme and of substrate satisfactorily, it is advisable to follow the time necessary to produce the same percentage change in substrate in the different cases. This was not done in the present instance, but, even so, some conclusions may be drawn from the results. Table VIII shows the values of K calculated by means of Schütz's rule from the equation

$$K = \frac{x}{\sqrt{E\,A}}$$

in which x represents the extent of the action, and E and A the relative concentrations of enzyme and substrate, respectively.

The average value of the constant of Schütz's rule from the results in Table VIII for Tumor Extract 19 is 4.4, for Tumor Extract 20 it is 4.5. There is quite a variation in the individual

values, and some of them are obviously incorrect. It is not easy
to draw any general conclusions as to possible trend of the values
with the limited number of results at hand, but perhaps it may
be said that the values in any one series tend to increase with
increased concentration of substrate. At any rate, in view of the
possible errors of the experiments and the use of amounts of action
in equal times instead of the more accurate use of lengths of time
for equal percentage actions, Schütz's rule indicates a rough
approximation to a probable explanation of the mechanism of the
actions. This is especially true when taken in connection with
the results of the time action determinations given in the pre-
ceding section, and points to the fact that some more general

TABLE VIII.

Values of the Constant of Schütz's Rule Derived from the Results of Table VII.

	Concentration of peptone.			
	1.0	2.5	5.0	10.0
Concentration of tumor extract 19.				
1	4.4	4.2	3.4	
2	3.7	4.4	5.2	
4	4.7	4.6	5.4	
Concentration of tumor extract 20.				
1	3.9	3.7	5.0	3.5
2	3.4	5.0	5.2	5.6
4	2.9	4.7	4.6	6.2

theoretical explanation of the mechanism of these enzyme actions
such as that outlined by Northrop is correct and that Schütz's
rule is a special and limited case of the general formulation.

*Influence of a Number of Neutral Salts on the Protease Actions
of Tumor Extracts.*—The protease actions of a number of tumor
extracts were tested after the addition of various neutral salts.
In place of giving the detailed experimental results for the indi-
vidual tests, the actions will all be given in relative terms, that is,
the percentage actions in the presence of the different concentra-
tions of the different salts with the action in the absence of the
salt in the given series as unity. In every series, an experiment
with the extract without added salt was run. The values of the
protease actions for these direct tests were, as a rule, of the same
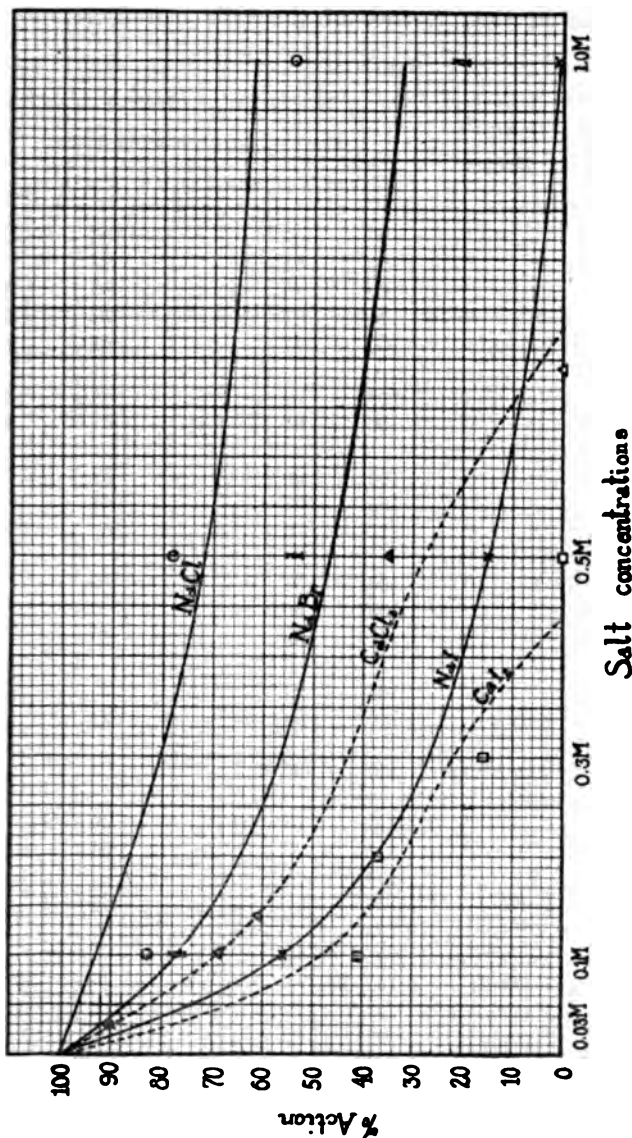
Fig. 3. Protease actions at pH 7.0 in the presence of salts. Concentrations of salts plotted as abscissæ; actions expressed as percentages of actions in the absence of salts, as ordinates.

general order of magnitude as the results already given. The relative actions in the presence of the salts can therefore be approximated.

TABLE IX.

Relative Protease Actions of Tumor Extracts in the Presence of Salts.

Salt.	Tumor extract No.	Method.	Molar concentrations of added salts.								
			0	0.03	0.10	0.14	0.20	0.30	0.50	0.69	1.0
NaCl	14	Van Slyke.	100		67				66		24
	18	" "	100		84				84		
	22	" "	100		85				85		53
	26	" "	100		84				72		69
	26	Formol.	100		94				84		70
NaF	14	Van Slyke.	100		76				55		48
	13	" "	100		95				95		42
NaBr	15	" "	100		62				52		0
	16	" "	100		92				48		0
	17	" "	100		76				62		64
NaI	15	" "	100		58				0		0
	16	" "	100		50				0		0
	17	" "	100		59				46		3
KCl	26	" "	100		91				73		65
	26	Formol.	100		94				84		61
LiCl	18	Van Slyke.	100		100				93		
CaCl₂	27	" "	100		58	50			26	0	
	19	" "	100	91					64		
	29	" "	100		58						
	27	Formol.	100				72		14	0	0
	29	"	100				75				
	30	Van Slyke.	100	79	55						
	30	Formol.	100	100	85						
	31	Van Slyke.	100		71						
	31	Formol.	100		82						
	32	"	100		69						
CaI₂	19	Van Slyke.	100		41		37	16	0		

The experiments were run in every case for 22 hours at 38°. Each final mixture contained the tumor in the concentration in-

dicated in Table I and 8 mg. of peptone per cc. Unless stated to the contrary, every tumor extract was dialyzed against tap water for 18 hours, and the mixtures were made up with these dialyzed solutions to which the salt, etc., were added. The final mixtures were made up to have a pH of 7.0.

The averages of the separate results for the different salts are given in Table X.

TABLE X

Average Values for the Relative Protease Actions of Tumor Extracts in the Presence of Salts.

Salt.	Molar concentrations of salts.							
	0	0.03	0.10	0.14	0.20	0.30	0.50	1.0
NaCl	100		83				78	54
NaF	100		86				75	45
NaBr	100		77				54	21
NaI	100		56				15	1
KCl	100		93				79	63
LiCl	100		100				93	
CaCl₂	100	90	69	61			35	0
CaI₂	100		41		37	16	0	

In order to facilitate comparison between the different actions, the results for sodium chloride, sodium bromide, sodium iodide, calcium chloride, and calcium iodide were plotted·and are shown in Fig. 3.

A study of the results given in Table IX shows considerable variations of the various tumor extracts under conditions of added salt where similar results, as expressed in terms of percentage actions, might be expected. In part, these variations are undoubtedly due to experimental errors, since the quantities measured are comparatively small. At the same time, variations between the influences exerted by different added substances on these protease actions would naturally be expected with tumors derived from different individuals, rat as well as human. Especially with human tumors of different types, as shown in the descriptions in Table I, would differences in actions be expected to occur. The data at hand are not extended enough to permit of a possible classification

of the actions according to the type and nature of tumor, so that for the purposes in view an average of the results for any one salt has been taken.   Under the circumstances, the general agreement of the relations between the effects of the added salts on the protease actions of the extracts of tumors from such very different sources is striking.

The average values for the actions of the various salts which are given in Table X clearly indicate the following facts:

1. The protease action was decreased in every case with increasing concentration of salt.

2. Sodium chloride and sodium fluoride exerted comparatively small retarding effects, the actions being decreased only one-fourth at 0.5 molar concentration, and one-half at molar concentration.

3. Potassium chloride and lithium chloride exerted smaller inhibiting actions, if anything, than did the sodium chloride and sodium fluoride, although the smaller number of results with the former do not make the results with them quite as satisfactory.

4. Sodium bromide retarded the actions to a greater extent than did sodium chloride, about one-fourth at 0.1 molar, one-half at 0.5 molar, and four-fifths at molar concentration.

5. Sodium iodide showed still greater retarding effects, the actions decreasing to about one-half at 0.1 molar concentration.

6. Calcium chloride retarded the actions to a much greater extent than the sodium chloride, but not as much as the sodium iodide for the corresponding molar concentrations.

7. Calcium iodide retarded the actions to the greatest extent of the salts which were studied.   This was to be expected in view of the retarding actions of sodium iodide and calcium chloride.

In view of the retarding actions of the calcium salts, the protease actions of some of the tumor extracts were determined after various treatments with calcium salts.   A definite time factor was observed.   The experiments in which the tumor extracts contained calcium chloride for some time before their activities were tested, showed markedly less protease actions on the peptone.

A study of the growth of transplanted Flexner-Jobling rat carcinoma after treatment with various solutions, will be spoken of in the following section.   In view of the relations found in this study, the protease actions of tumor extracts in the presence of salts mixed in the proportions used in the Locke-Ringer solution

were determined. Table XI gives the results of these determinations with the salts present in the concentrations given in such a solution, in three times these concentrations, and also in a solution containing only the sodium chloride. The results are presented as percentage actions, with no added salt present taken to be 100.

TABLE XI.

Relative Protease Actions of Tumor Extracts in the Presence of Salt Mixtures.

Experiment No.	Method.	No salt.	0.15 M NaCl.	0.45 M NaCl.	0.15 M NaCl 0.003 M CaCl₂ 0.003 M KCl	0.45 M NaCl 0.009 M CaCl₂ 0.009 M KCl
34	Van Slyke.	100		55	69	64
	Formol.	100	86	64	100	78
35	Van Slyke.	100	72	82	94	
	Formol.	100	99	83	100	85
36	Van Slyke.	100	97	76	89	75
	Formol.	100	86	69	91	72
39	Van Slyke.	100	73	82	89	74
	Formol.	100		86		93
Averages........		100	86	75	90	77

Calcium chloride, in the concentrations 0.003 and 0.009 M, did not retard the actions. In fact, a slight increase was noticed as compared with the actions in the absence of the salt, but this may have been due to experimental inaccuracies. The results shown in Table XI indicate clearly that the addition of calcium and potassium chlorides to the sodium chloride solution in the relative proportions of the Locke-Ringer solution, did not reduce the retarding action of the sodium chloride on the protease actions. The concentrations of the calcium and potassium chloride were too small to cause appreciable retarding actions in themselves. A comparison of these results with the results of Tables IX and X and with the corresponding curves in Fig. 3 brings this out clearly. In other words, there is no antagonistic salt action observable with the protease of tumor extracts.

DISCUSSION OF RESULTS.

The discussion of the experimental results involves: first, a comparison of the protease actions of the tumor extracts with the actions of proteases from different sources; and second, a comparison of the tumor protease actions with transplantation phenomena of the Flexner-Jobling rat carcinoma.

The protease actions found do not differ in any significant way from the protease actions observed by other workers with material from similar sources.[9] It must be remembered that the present investigation did not include directly the study of the chemical composition of tumors.

An important point which should be emphasized is that as far as these studies went, the protease actions of human and rat tumor extracts were essentially the same. This applies especially to the optimum hydrogen ion concentration for the actions and to the behavior of neutral salts, the inhibiting effect of calcium in both series being striking. The tumor tissue protease was found to be somewhat more active than the normal tissue protease. This agrees with the observation of Abderhalden,[10] who also found that extracts of normal and of tumor tissue of rats and mice hydrolyzed polypeptides and silk peptone in different ways. However, it may be recalled that enzymes of normal tissue from different parts or organs of the same animal may well produce very different results on the same substrate.

The pH value for optimum protease action was found to be 7.0 with more rapid decrease on the acid than on the alkaline side, as shown in Fig. 1. The action may be said to be tryptic in character.[11] The hydrogen ion concentration for the optimum action is very nearly that of the blood and probably of most of the tissues. As far as can be told from the results obtained, the kinetic relationships are similar to those observed by others as already pointed

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[9] *Cf.* for example Wells, H. G., Chemical pathology, Philadelphia, 2nd edition, 1914, 456–8. Ewing, J., Neoplastic diseases, Philadelphia, 1919, 90–1.

[10] Abderhalden, E., and Medigreceanu, F., *Z. physiol. Chem.*, 1910, lxvi 265. Abderhalden, E., and Pincussohn, L., *Z. physiol. Chem.*, 1910, lxvi, 277.

[11] *Cf.* Loeper, G. F., and Tannet, G., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 993; *J. pharm. et chim.*, 1920, xxii, 210.

out.   The facts that the substrate in such actions is not a simple one, and that probably several different reactions are occurring, either simultaneously or consecutively, serve to obscure the actual relationships of the actions.   In general terms, the actions are of the same type as other protease actions.

The relation between the protease actions and the growth of transplanted tumors may be summarized briefly.

The growth of the transplanted Flexner-Jobling rat carcinoma was followed, the tissue fragments before inoculation being immersed for various lengths of time in solutions of different hydrogen ion concentrations and salt contents.   The details of this work are presented elsewhere.[12]   The following conclusions are of interest in the present connection.   After immersion in phosphate mixtures of different hydrogen ion concentrations for 24 hours in the ice box, the transplants immersed in the pH 7.0 solutions grew normally, while those in the more acid and more alkaline solutions did not grow.   Immersion in physiological salt solution (0.15 M NaCl) at pH 7.0 for 24 hours did not prevent growth, with immersion for 72 hours, however, no growths were obtained. With calcium chloride solutions (0.078 M CaCl$_2$), growth was prevented in much shorter times of immersion, 10 hours or less.   The inactivating or harmful effects of the sodium chloride in the longer period of immersion, or of a much more dilute calcium chloride solution in a correspondingly longer time were overcome by the use of a "balanced" mixture of the two salts.   Thus, immersion in a solution of 0.15 M NaCl and 0.003 M CaCl$_2$ concentration for 72 hours showed normal growths of the transplants.

Summarizing the comparison of the protease actions of the aqueous extracts of human and rat tumors and the growth of the Flexner-Jobling rat carcinoma after immersion in different solutions before transplantation, it may be stated that the same optimum or favorable acidity of the solution is necessary for both (in the neighborhood of pH 7.0), that sodium chloride and calcium chloride exert retarding, inhibiting, or harmful actions on both sets of phenomena, calcium chloride much more so than sodium chloride for corresponding concentrations.   On the other hand, in the tumor transplants, antagonistic salt action was

[12] Sugiura, K., Noyes, H. M., and Falk, K. G., *J. Cancer Research*, 1921, vi. in press

observed with sodium chloride and calcium chloride, while in the enzyme actions the salt effects were not antagonistic but appeared to be additive.

The two sets of actions, although paralleling each other in certain respects, are evidently to be ascribed to different causes. With the tumor transplants, the behaviors are connected with the actions of the salts on the cell membranes and the changes in permeability of the latter. With the enzyme actions, the effects appear to be dependent upon a more direct interaction between the salt and the enzymically active molecule or grouping. At the same time, when it is considered that growth is connected with, and possibly dependent upon, enzyme actions, even the partial parallelism of the harmful effects of the different conditions upon the cell membranes and upon the actions of the enzymes within the cells, is of interest and perhaps of some significance.

SUMMARY.

The proteolytic actions on casein and on peptone of extracts of malignant human tumors and of the Flexner-Jobling rat carcinoma were studied by the Van Slyke amino nitrogen and the Sörensen formol methods at different hydrogen ion concentrations, in the presence of a number of neutral salts, for various lengths of time, and with various concentrations of enzyme and of substrate.

Similar results were obtained with the extracts from the two sources. Optimum conditions for action were found at pH 7.0. The general actions were similar to those of other protease preparations and could be formulated similarly. Tables and curves showing the retardations exerted by various neutral salts and by mixtures of several salts are given.

STUDIES OF URINARY ACIDITY.

I. SOME EFFECTS OF DRINKING LARGE AMOUNTS OF ORANGE JUICE AND SOUR MILK.

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It is known that the eating of most fruits leads to the formation of less acid urines due to the excess of base-forming elements in the fruits. The organic acids contained in the various fruits are oxidized within the body to carbon dioxide and water, so that the net result to the organism is a gain in base. However, fruits such as cranberries, prunes, and plums, which contain benzoic acid or its precursors, cause the production of more acid urines. This result is, doubtless, in part due to the synthesis of hippuric acid (Blatherwick, 1914).

During the progress of a study of the neutrality regulation of cattle, it was observed that a cow fed large amounts of corn silage, a product with an excess of base-forming elements, excreted an acid urine and showed a reduction in the plasma carbon dioxide-combining power (Blatherwick, 1920). The belief was then expressed that these effects were probably caused by the incomplete oxidation of the contained organic acids. The organic acids of corn silage are chiefly racemic lactic, acetic, and butyric, (Dox and Neidig, 1912, 1913). In view of the foregoing, the question arose as to whether the tolerance of man for naturally occurring fruit acids could also be passed. Orange juice was chosen for study because of its wide-spread use, and owing to the observation of Newburgh and Marsh (1920) that one of their diabetic patients succumbed after eating a bag of oranges.

Plan of Orange Juice Experiment.

Two healthy young women volunteered as subjects for this study. Subject O was given a uniform basal diet of the following composition; baked potato, 260 gm.; whole milk, 440 gm.; graham crackers, 300 gm.; raw apple, 150 gm.; cheese, 25 gm.; butter, 45 gm.; and egg, 65 gm. The diet of Subject L was similar with the exception that she ate no egg. After 4 days on this basal diet the effect of increasing amounts of strained orange juice was determined.

Methods.

The following analytical methods were used; pH, Palmer, Salvesen, and Jackson (1920–21); organic acids, Van Slyke and Palmer (1920); ammonia, Folin and Bell (1917); nitrogen, Folin and Wright (1919); and CO_2 capacity of plasma, Van Slyke (1917).

Discussion of Results with Orange Juice.

In Table I are shown the analytical data obtained from the the urines of the two subjects for the basal and experimental diets. The effects on the urine of drinking large amounts of orange juice are the following: a marked increase in the pH value (less acidity); an increased excretion of organic acids; and a marked decrease in the ammonia output. This increase in the organic acid excretion with a coincident decrease in the ammonia content is interesting because it illustrates an exception to the general rule of parallelism of these two factors. The explanation of this peculiar result is probably the following: a certain part of the citric acid escapes oxidation and is eliminated in the urine as citrate and thus increases the titration value for organic acids, while the excess of base in the orange juice is sufficient to more than balance the organic acidity and to cause a marked depresssion in the ammonia excretion. The values given by Sherman show that the ash of orange juice contains an excess of base amounting to 4.5 cc. of normal solution per 100 gm. of juice. Therefore, on the last experimental day, the value for excess base from the orange juice, corresponded with 1,080 cc. of normal solution. Determination of the organic acid content of orange juice by the method of Van Slyke and Palmer indicated a concentration of 1.84 per cent citric acid. Of this amount, 59 per cent appeared to be present as the free acid when titrated with 0.1 N NaOH,

TABLE I

Composition of Urine, and Plasma Carbon Dioxide Capacity.

Date.	Urine.					Plasma.	Remarks.
	Volume.	pH	0.1 N organic acids	Ammonia N	N	CO₂ capacity	

Subject O.

1921	cc.		cc.	gm.	gm.	cc.	
Nov. 4	875	6.8	507	0.26	7.79		Basal diet.
" 5	710	6.8	562	0.26	7.16		" "
" 6	720	6.6	522	0.25	7.06		" "
" 7	720	7.1	599	0.21	7.09		" "
" 8	1,355	7.1	777	0.19	8.35	63.3	Basal diet + 600 cc. orange juice.
" 9	1,750	7.1	647	0.16	7.35		" " +1,200 " " "
" 10	2,290	7.2	914	0.12	7.56		" " +1,800 " " . "
" 11	3,160	7.4	929	0.13	8.21		" " +2,400 " " "
" 12						62.4	

Subject L.

Nov. 4	1,210	6.4	488	0.21	6.07		Basal diet.
" 5	780	6.6	498	0.14	5.27		" "
" 6	610	6.9	512	0.13	5.98		" "
" 7	905	6.9	494	0.13	6.20		" "
" 8	1,700	6.9	585	0.15	6.29	59.5	Basal diet + 600 cc. orange juice.
" 9	2,250	7.3	699	0.10	5.67		" " +1,200 " " "
" 10	2,830	7.2	844	0.10	5.94		" " +1,800 " " "
" 11	2,550	7.4	878	0.07	6.98		" " +2,400 " " "
" 12						63.3	
						67.2	1 hour after drinking 800 cc. orange juice.

using phenolphthalein as an indicator. Since by this method, citric acid is titrated to the extent of about 90 per cent, the true value for total citric acid would be about 2.0 per cent. The increased excretion of organic acids in the urines of the last day above the average excretion of the preliminary period amounted

to 382 and 380 cc. of 0.1 N acid. Assuming that these increases were entirely due to citric acid, an escape of 2.71 gm. of this acid into the urine is indicated. Therefore, approximately 6 per cent of the ingested citric acid appears to have escaped oxidation. Salant and Wise (1916) showed that after hypodermic injection of sodium citrate in rabbits about 12 per cent was excreted in the urine; with cats 30 per cent, and with dogs 40 per cent.

Observations of the plasma carbon dioxide capacity of Subject O showed practically no change as the result of drinking large amounts of orange juice. However, the plasma of Subject L gave an increase from 59.5 cc. before the orange juice period to 63.3 cc. at the close. On November 12th, this subject drank 800 cc. of orange juice at one time. Determinations of plasma CO_2 capacity showed values of 63.3 cc. before and of 67.2 cc. 1 hour afterward.

The foregoing results seem to render it improbable that the ingestion of large amounts of citrous fruits or fruit juices is capable of causing the production of an acid urine by overstepping the organism's ability to oxidize the organic acid or acids contained therein. (The results, of course, do not apply to fruits which yield acids other than citric in the body.) The subjects on the last day each drank 2,400 cc. or the equivalent of about 24 large oranges, containing approximately 48 gm. of citric acid. One may, therefore, feel secure in eating unlimited amounts of oranges without fear of acidotic effects.

Lactic Acid Experiments.

A basal diet of 2 liters of whole milk and 340 gm. of soda crackers was selected. This diet amply satisfies the ordinary requirements and is one which should theoretically yield a neutral urine. On the experimental days 2 liters of the lactic acid milk were substituted for the whole milk. This arrangement permitted a constant intake of the ash constituents throughout the experiment.

In addition to the determinations performed in the citric acid experiments, phosphorus was determined by the colorimetric method of Bell and Doisy (1920).

The experimental milk was prepared by inoculating skimmed milk with a lactic acid-producing culture and allowing it to ferment for 12 hours at 37° C. Such milk then showed an organic acid content of approximately

2 per cent, expressed as lactic acid, as determined by the method of Kramer and Greene (1921). The product at this point was full of large clumps of coagulated material which were next thoroughly broken up. Just before serving the calculated amount of cream was added to bring the fat content to that of whole milk.

RESULTS AND DISCUSSION.

Reference to Table II shows that the effects upon the urine of drinking large amounts of lactic acid milk were: a marked decrease in pH (increased acidity); a marked increase in the titratable acidity; a marked increase in the phosphorus content; a significant increase in the ammonia output; and no change in the organic acidity. These changes occurred in both subjects.

Determinations of the plasma CO_2 capacity led to rather variable results. Subject Ot. experienced no decrease in plasma CO_2 capacity upon changing from whole milk to sour milk but did show a marked decrease 1 hour after drinking 500 cc. of the acid milk. On the other hand, Subject Ol. responded with a decrease in the change from whole milk to the acid milk but showed no change as the result of drinking 500 cc. of this product. All in all, these findings may be taken as evidence that the ingestion of such large amounts of lactic acid are capable of reducing the alkaline reserve by the entrance of the acid into the blood. Our results are similar to those recently reported by Taistra (1921) who observed a decrease in plasma CO_2 capacity and an increase in the titratable acidity of the urine of a dog which was fed a meat broth containing lactic acid.

The mechanism involved in the production of the increased acidity of the urine resulting from the ingestion of lactic acid is very interesting. The increased output of acid in our experiments was caused by an augmented excretion of acid phosphate, as indicated by simultaneous increases in the titrable acidity and in the phosphorus content of the urines. None of the increased acidity was due to the organic acid fraction, that is, the lactic acid was completely oxidized or retained. The entrance of lactic acid into the blood stream seems to have evoked a compensatory elimination of acid phosphate by the kidney. The increased acidity of the urines resulting from the eating of fruits containing benzoic acid, and the strongly acid urines of diabetic patients may

perhaps be produced in a somewhat analagous manner.   Further study along these lines is in progress.

TABLE II.

Composition of Urine, and Plasma Carbon Dioxide Capacity.

Date.	Urine.							Plasma.	Remarks.
	Volume.	pH	Titratable acidity.	0.1 N organic acids.	P.	Ammonia N	N	CO₂ capacity.	

(subscript: CO$_2$ capacity)

Subject Ot.

1922	cc.		cc.	cc.	gm.	gm.	gm.	cc.	
Jan. 23	1,480	6.5	218	453	0.77	0.39	12.64		Whole milk diet.
" 24	1,590	6.2	313	516	0.88	0.46	14.18		" " "
" 25	2,040	5.5	433	524	1.00	0.56	13.46	56.7	Acid milk diet.
" 26	2,270	5.7	391	547	1.06	0.60	13.03		" " "
" 27	1,830	6.5	200	457	0.84	0.42	11.87	57.6	Whole milk diet.
" 28								58.5	Before drinking 500 cc. acid milk.
								51.8	1 hour after drinking 500 cc. acid milk.
								59.5	3 hours after drinking 500 cc. acid milk.

Subject Ol.

Jan. 23	1,480	6.9	121	501	0.65	0.43	11.01		Whole milk diet.
" 24	2,000	6.7	268	539	0.99	0.48	14.16		" " "
" 25	1,620	5.6	516	469	1.28	0.48	14.09	66.2	Acid milk diet.
" 26	2,225	5.9	444	554	1.22	0.56	14.11		" " "
" 27	2,090	6.8	220	557	1.00	0.46	13.65	59.5	Whole milk diet.
" 28								61.4	Before drinking 500 cc. acid milk.
								62.4	1 hour after drinking 500 cc. acid milk.
								62.4	3 hours after drinking 500 cc. acid milk.

SUMMARY.

The drinking of large amounts of orange juice resulted in the production of alkaline urines, an increased organic acid excretion,

and a decreased ammonia content of the urines.   It was impossible to overreach the organism's ability to oxidize the contained citric acid even though the amounts drunk in 1 day were the equivalent of about 48 gm. of acid.

The drinking of large amounts of lactic acid milk caused the formation of strongly acid urines. This increased acidity was shown to be due to the excretion of increased amounts of acid phosphate, as indicated by simultaneous increases in titratable acidity and phosphorus. The lactic acid appeared to have been completely oxidized or retained, as there was no change in the organic acid excretion.

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THE FERMENTATION OF HEXOSES AND RELATED COMPOUNDS BY CERTAIN PENTOSE-FERMENTING BACTERIA.*

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The fermentation products of the hexoses usually differ from those of the pentoses. A common product, such as lactic acid, is generally produced from both types of sugar, but the difference usually lies in the other major products. The constancy with which lactic acid is formed from such different compounds as glucose and xylose indicates that three of the carbon atoms contained in the sugar molecules appear as lactic acid, while the residuum is converted into a two- or three-carbon compound depending upon the kind of sugar and the type of organism attacking it. In some cases this residuum is transformed into such products as acetic acid, ethyl alcohol, and carbon dioxide, singly or in pairs.

A cleavage of the molecules between the third and fourth carbon atoms counting from the alcohol end of the chain is indicated. The basis for this conclusion rests on the similarity of this portion of the structural formula of such different compounds as xylose, glucose, fructose, and mannitol, and the differences that obtain at the other end of their structural formulæ. A two-carbon residue, an aldehyde, a ketone, or an alcohol group is a potent factor in the determination of the end-products formed.

Cleavage along certain well defined lines, and constant intermediate products are indicated by such results. By a quantitative

determination of the end-products of fermentation and a study of these data, it is possible that some rational theory such as exists for the action of yeast, may be evolved concerning the mechanics of carbohydrate fermentation by bacteria.

In previous publications (1, 2) we have described the end-products formed from hexoses and related compounds by certain pentose-fermenting bacteria which were designated as *Lactobacillus pentoaceticus*. From the aldo-hexoses, lactic acid, ethyl alcohol, and carbon dioxide are formed approximately in the proportions required by the equation:

$$C_6H_{12}O_6 = C_3H_6O_3 + C_2H_5OH + CO_2$$
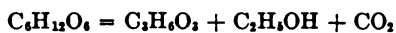
From fructose, acetic acid is formed instead of ethyl alcohol, and as a corrolary to the main fermentation, a reduction product, mannitol, is produced. From the quantitative data, the following equations may be deduced to represent the fermentation of fructose:

(1) $C_6H_{12}O_6 + H_2O = C_3H_6O_3 + C_2H_4O_2 + CO_2 + 2H_2$

(2) $2C_6H_{12}C_6 + 2H_2 = 2C_6H_{14}O_6$
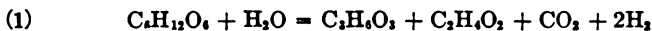
Two products of fermentation, carbon dioxide and mannitol, are the most distinctive compounds for differentiating *Lactobacillus pentoaceticus* from another group of pentose-fermenters which have been described in a more recent publication (3). The latter form no mannitol and produce only traces of carbon dioxide. Coincident with the absence of carbon dioxide is their failure to produce ethyl alcohol or acetic acid from the hexoses. If the production of ethyl alcohol and carbon dioxide be taken as indicative of the enzyme, carboxylase, the first group of bacteria may be described as carboxylase-positive and mannitol-forming, and the second group as carboxylase-negative and non-mannitol-forming organisms.

The members of the second group are in general somewhat more vigorous fermenters, in that they attack a greater variety of compounds, and destroy a larger percentage of the compound attacked. They are less sensitive to changes in the reaction of the medium, and operate through a wider range of hydrogen ion concentration. The first group is inhibited by a slight alkalinity

while the second is able to grow in a fairly alkaline solution. The second group forms lactic acid from both halves of the sugar molecule, while the first group converts one-half of the molecule to ethyl alcohol (or acetic acid) and carbon dioxide. Lactic acid represents from 90 to 95 per cent of the sugar consumed by the second group, and accordingly the fermentation of the hexoses may be represented by the simple equation

$$C_6H_{12}O_6 = 2C_3H_6O_3$$

The disaccharide, lactose, and the trisaccharides, raffinose and melezitose, are fermented to the same end-product. In old cultures it is probable that a secondary fermentation of the lactic acid ensues with the production of acetic acid, traces of formic, and possibly ethyl alcohol and carbon dioxide.

The formation of lactic acid as the sole major end-product of fermentation relates these bacteria closely to the group of milk organisms designated *Streptococcus lactis* Lister. As a means of comparison, a representative of this group has been included in these experiments. A distinctive difference between the two, however, is the inability of the *Streptococcus lactis* group to ferment pentoses, which is the most conspicuous property of the organisms used for the fermentations discussed in this paper.

EXPERIMENTAL.

The kind of culture medium, the type of fermentation flask, and the methods of analysis for the different fermentation products have been described in previous papers (1). In most of the fermentations the acids formed were neutralized by the addition of sterilized 1.0 N sodium hydroxide. When the fermentable substance was not vigorously attacked as in the case of lactose and mannitol, better fermentation was obtained by keeping the solutions neutral with an excess of calcium carbonate. The improved results are no doubt due to a more favorable hydrogen ion concentration. The disadvantage in the use of calcium carbonate is that the carbon dioxide evolved by the bacteria cannot be determined when this salt is added to the medium.

Four cultures of pentose-fermenting bacteria, Nos. 29, 124-2, 102, and 31, and a strain of *Streptococcus lactis* have been used in

this work. Cultures 102 and 31 are of especial interest because
they ferment arabinose, but do not attack xylose. Cultures 29
and 124-2 are much alike in their fermentative powers, but differ
from Cultures 102 and 31 in that they are unable to ferment the
trisaccharide, melezitose. *Streptococcus lactis* ferments neither
melezitose nor raffinose.

Because of the labor involved and because certain of the sugars
are not fermented, complete data for each sugar with all these
bacteria are not given. It was decided to limit the fermentations
to glucose, fructose, lactose, raffinose, and melezitose, as repre-
sentative of the sugars, and to mannitol as representative of
the hexahydric alcohols. Since the sugars used furnish all the
monosaccharides obtained from maltose and sucrose it is felt that
nothing of great importance would have been obtained by including
these two disaccharides.

Fermentation Products.

The weight of sugar fermented, the quantity of products formed,
and the relation of these products to the sugar consumed are given
in Table I. The data show the extensive fermentation of all the
sugars, practically complete in many cases and rarely less than
80 per cent. As a rule less than 0.1 gm. of sugar remained un-
fermented and sometimes as little as 0.03 gm. in 100 cc. of culture.
As judged by the rate of acid production, Culture 124-2 was per-
haps the most vigorous fermenter. The milk organism *Strepto-
coccus lactis* was noticeably slower and less extensive than the
pentose-fermenters; from 0.1 to 0.4 gm. of sugar remained un-
fermented in 100 cc. of culture.

Acids.—The sugars are fermented almost quantitatively to
lactic acid, which product represents from 90 to 95 per cent of the
sugar consumed. Volatile acid was not formed from the mono-
saccharides, but appreciable quantities were produced from lac-
tose. Because of the slow rate of destruction of lactose, the
fermentations were allowed to continue for from 40 to 60 days.
No sugar was present at the end of this time, and it is probable
that the bacteria were forced to use lactic acid as a source of
energy. In the case of *Streptococcus lactis* no volatile acid was
formed, due perhaps to the presence of sugar; 0.297 gm. remained
even after 44 days. From the trisaccharides, only traces of

volatile acid were formed, possibly because of the difficulty with which these sugars are attacked, and as a consequence some of the lactic acid is converted into volatile acid. The production of volatile acids from lactic acid has been noted by several investigators (2, 4).

TABLE I.

Total Fermentation Products from Sugars.

Culture No.	Age of culture.	Carbohydrate.	Calculated for 100 cc. of culture.				
			Weight of compound fermented.	Volatile acid as acetic acid.	Non-volatile acid as lactic acid.	Carbon dioxide.	Weight of compound accounted for by products.
	days		*gm.*	*gm.*	*gm.*	*gm.*	*per cent*
29	10	Glucose.	1.831	0.000	1.675	0.032	94
124–2	11	"	1.839	0.000	1.706	0.030	94
102	13	"	1.805	0.000	1.637	0.024	92
31	13	"	1.784	0.000	1.703	0.030	97
S. lactis.	15	"	1.790	0.000	1.599	0.020	91
29	10	Fructose.	1.620	0.000	1.434	0.023	90
·124–2	10	"	1.635	0.000	1.510	0.019	95
102	8	"	1.620	0.000	1.520	0.026	95
31	8	"	1.586	0.000	1.434	0.030	92
29	15	Lactose.	1.335	0.000	1.211	0.045	91
29	63	"	2.036	0.078	1.807	Undetermined.	92
124–2	63	"	2.036	0.101	1.799	"	88
102	44	"	2.036	0.065	1.817	"	92
S. lactis.	44	"	1.785	0.000	1.578	"	86
29	24	Raffinose.	1.630	0.018	1.478	0.077	95
102	24	"	1.610	0.031	1.397	0.062	91
102	16	Melezitose.		0.010	1.641	0.049	
31	16	"		0.009	1.646	0.047	

It is noteworthy that the pentose-fermenters and the milk organism fermented the different sugars used in much the same way. The two types are, however, very different morphologically, and in their ability to ferment certain carbohydrates; the pentose-

fermenters attack a much larger number of compounds than *Streptococcus lactis*. In the cases where both ferment a given compound, the products seem to be alike and in about the same proportions. Another characteristic difference is the kind of lactic acid formed. The inactive form of lactic acid was produced by the pentose-fermenters while *Streptococcus lactis* always produced the active form.

Carbon Dioxide.—The carbon dioxide is too small to represent a direct fermentation product. From 20 to 80 mg. produced by the fermentation of approximately 2 gm. of sugar can scarcely be regarded as a fermentation product. It is more reasonable to assume this to represent the respiration of the cells. It may be regarded as a product of the endogenous metabolism of the cells; *i.e.*, the catabolic processes of cellular tissue. In this connection it should be noted that the amount of carbon dioxide produced from the pentoses, xylose and arabinose, by these organisms is essentially the same as that produced from the hexoses and their related compounds.

Fermentation of Mannitol.

The fermentation of mannitol is quite different from that of the sugars. These are either aldehydes or ketones while mannitol is an alcohol. No alcohol or volatile acid was obtained from the sugars while both of these products were found in the mannitol cultures. In every case ethyl alcohol was formed in the breaking down of mannitol, and in some of the fermentations in considerable amounts, from 8 to 16 per cent of the total end-products. Volatile acids which later will be shown to be formic and acetic were produced by all of the bacteria with the exception of No. 102. As in the case of alcohol, the volatile acids increase with the age of the culture. The lactic acid, on the other hand, decreases in the older cultures. These changes suggest a secondary fermentation of lactic acid to alcohol, formic acid, and acetic acid. Mazé (4) has shown that such a fermentation is possible. More recently Aubel (5) reported these same products in the fermentation of pyruvic acid. The rather difficult fermentation of mannitol forcing the organism to attack the lactic acid lends support to this hypothesis. On the other hand, the absence of alcohol from some of the sugar fermentations where lactic acid was apparently destroyed

is evidence against this view. A direct relation between alcohol and mannitol appears certain. The stereoisomeric structure of the compounds seems to determine the nature of the end-products. An alcohol group results in the formation of ethyl alcohol while an aldehyde or ketone group eventuates into lactic acid.

Certain differences in the fermentation products of the various bacteria manifest themselves. The pentose-fermenter, No. 102, never forms volatile acid while *Streptococcus lactis* forms conspicuously large amounts. Cultures 29 and 124-2 are large producers of both alcohol and volatile acid. The data are given in Table II.

TABLE II.

Total Fermentation Products from Mannitol.

Culture No.	Age of culture.	Calculated for 100 cc. of culture.			
		Volatile acid as acetic acid.	Non-volatile acid as lactic acid.	Alcohol as ethyl.	Carbon dioxide.
	days	*gm.*	*gm.*	*gm.*	*gm.*
29	68	0.116	1.041	0.211	
29	38	0.000	1.209	0.011	
124-2	68	0.143	0.553	0.139	
124-2	38	0.085	0.991	0.129	
102	68	0.000	1.335	0.154	
102	38	0.000	0.364	0.064	
S. lactis.	68	0.182	0.336	0.042	
" "	38	0.070	0.427	0.047	
29	20	0.016	0.359	0.037	0.024
124-2	20	0.027	0.398	0.265	0.034
102	20	0.003	0.634	0.028	0.033
S. lactis.	20	0.130	0.438	0.052	0.034

Forms of Lactic Acid Produced.

Many investigators (6) have determined the optical form of lactic acid produced by different bacteria. Levo, dextro, and inactive forms have all been produced by pure cultures. Some investigators have even claimed that the type of acid produced varies with the conditions of growth, but these claims rest on rather doubtful evidence.

In all of our work on pentose-fermenters, only inactive lactic acid has been found. The possibility of a small amount of an

active form has been considered but hitherto no attempt has been
made to isolate such a form.   To determine if any such form were
present, the lactic acid obtained from glucose was subjected to
fractional crystallization.   As the active lactic acid is more soluble
than the inactive type, fractional crystallization would separate
the two salts.   As a basis for comparison and as a check on the
procedure, the lactic acid formed by *Streptococcus lactis* was in-
cluded in the analysis.

The entire quantity of lactic acid produced by each culture was
converted into zinc lactate and separated into three fractions.
The different fractions were analyzed for their water of crystalli-

TABLE III.

Water of Crystallization of Zinc Lactates Obtained from Glucose.

Culture No.	Crop No.	Weight of salt.	Loss on heating.	Water of crystallization.	Theory.
		gm.	*gm.*	*per cent*	*per cent*
124-2	1	3.3474	0.6032	18.02	18.1
124-2	2	0.5066	0.0884	17.47	18.1
124-2	3	0.1290	0.0234	18.14	18.1
29	1	1.9988	0.3618	18.10	18.1
29	2	0.8074	0.1440	17.83	18.1
29	2a*	0.7164	0.1294	18.06	18.1
S. *lactis.*	1	2.0970	0.2682	12.80	12.8
" "	2	0.3084	0.0396	12.84	12.8
" "	3	0.1284	0.0160	12.46	12.8

* Crop 2 recrystallized.

zation to determine if there was any departure from the theoretical
amount.   If both active and inactive zinc lactate were present,
the inactive form would come out mainly in the first crop
while the active form would appear largely in the last crop.   No
differences in the water of crystallization of the various fractions
occurred, and all agreed well with the theoretical value.

The pentose-fermenters formed inactive lactic acid only, and
Streptococcus lactis produced only the active form.   The weights
of zinc lactate and the loss of water on heating, together with the
percentage of water of crystallization are given in Table III.

The water of crystallization for the zinc lactate produced from
all the compounds fermented by the various bacteria are given

in Table IV.   The blank spaces are due, either to the fact that a fermentation was not made, as in the case of *Streptococcus lactis* on fructose, or because the particular sugar is not fermentable by the given bacteria; for example, Cultures 29 and 124-2 do not attack melezitose.   The pentose-fermenters produced inactive lactic acid from all the different sugars and mannitol, while the lactic acid organism from milk just as uniformly produced the active enantiomorph.

The kind of active lactic acid produced by *Streptococcus lactis* was ascertained by determining the specific rotation of the zinc

TABLE IV.

*Forms of Lactic Acid Produced as Determined by the Water of Crystallization of Their Zinc Salts.**

Source of lactic acid.	Crop No. of salt.	Culture 29.	Culture 124–2.	Culture 102.	Culture 31.	S. lactis.
		per cent	per cent	per cent	per cent	per cent
Glucose.	1	18.1	18.0	18.0	18.0	12.80
"	2	17.8	17.5	17.7	17.7	12.84
"	2a	18.1		18.2		
"	3		18.1			12.46
Fructose.	1	18.1	18.2	18.1	18.1	
Lactose.	1	18.0	18.1	18.1		12.9
Raffinose.	1	18.2		18.2		
Melezitose.	1			18.0	18.1	
"	2			17.9	17.9	
Mannitol.	1	18.0	18.2	18.2		13.1

* Inactive zinc lactate contains 18.17 per cent and active zinc lactate 12.8 per cent water of crystallization.

salts.   The zinc lactate from lactose was recrystallized, and 1.2366 gm. of the anhydrous salt were dissolved in 30 cc. of water at 22°C.   The rotation produced at this temperature in a 2 dm. tube was found to be $-1.83°$ on the Ventzke scale.   When calculated to the corresponding specific rotation, the figure -7.67 was obtained.   The value obtained in the same way for the zinc lactate from the fermentation of glucose was -7.63.   Hoppe-Seyler and Araki (7), with comparable concentrations and temperature, found active zinc lactate to possess a specific rotation of ±7.522.   Since the rotation of the zinc salt is the opposite of the free acid, it is evident that this culture of *Streptococcus lactis* produced *d*-lactic acid.

Identification of Volatile Acids and Alcohol.—The volatile acid obtained directly from the culture and that resulting from the oxidation of the alcohol were subjected to a Duclaux distillation, and the distilling constants calculated from the titration data. The constants obtained together with that for pure acetic acid, are given in Table V. The constants for the acids resulting from the oxidation of the alcohol from mannitol and volatile acid from lactose are in good agreement in all cases with that for acetic acid, and hence indicate that ethyl alcohol and acetic acid are produced from mannitol and lactose, respectively. The constants for the volatile acid from mannitol lie between those given by Duclaux for acetic and formic acids. It is quite evident that the volatile acid is a mixture. Since no qualitative tests indicated the presence of propionic acid, it was assumed that the mixture consisted only of formic and acetic acids. From the distilling constants the percentages of the two acids were calculated by the graphic method of Gillespie and Walters (8) and are probably correct to within 5 per cent.

The results were found to be as follows:

Culture No.	Formic acid.	Acetic acid.
	per cent	*per cent*
29	70	30
124–2	61	39
S. lactis.	50	50

As a check on the Duclaux data, the percentage of formic acid was determined by Fincke's method (9). An immediate and heavy precipitation of mercurous chloride left no doubt of the presence of formic acid. The data are given in Table VI. A comparison of the percentages of formic acid found by the two methods shows good agreement in all but one case. There can be no question that formic and acetic acids are produced in the fermentation of mannitol by these different bacteria. It is not easy, however, to visualize the mechanics of this fermentation whereby lactic acid is the major product, and ethyl alcohol, formic acid, acetic acid, and possibly small quantities of carbon dioxide, and even hydrogen are minor products. With more extensive data it appears probable that correlative processes of reduction and oxidation will unfold the steps in the formation of these biochemical products.

TABLE V.

Distilling Constants of the Volatile Acids Obtained by the Duclaux Method.

Culture No.	Source of acid.	Fractions.									
		10 cc.	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.	80 cc.	90 cc.	100 cc.
29	Lactose.	7.6	15.4	23.5	31.8	40.8	50.3	60.5	71.5	84.3	100
124–2	"	7.6	15.5	23.6	32.0	40.6	50.3	60.5	71.7	84.2	100
102	"	7.4	15.1	23.2	31.7	40.7	50.1	60.2	71.4	84.3	100
29	Mannitol.	6.2	13.2	20.4	28.1	36.5	45.6	55.8	67.5	81.5	100
124–2	"	6.7	13.9	21.4	29.2	37.7	46.8	56.8	68.2	81.8	100
S. lactis	"	6.7	13.8	21.6	29.6	38.1	47.3	57.3	68.9	82.2	100
29	Alcohol from mannitol.	7.7	15.8	23.0	32.8	42.0	51.6	61.8	72.9	85.3	100
124–2	" "	7.7	15.5	23.9	32.9	42.0	51.7	61.9	73.0	85.4	100
102	" "	7.6	15.7	24.1	32.8	41.9	51.5	61.8	72.9	85.4	100
Duclaux constant for acetic acid.		7.4	15.2	23.4	32.0	40.9	50.5	60.9	71.9	84.4	100

TABLE VI.

The Formic Acid Content of the Volatile Acids Produced from Mannitol.

Culture No.	Total acid in aliquot as 0.1 N.	Weight of HgCl.	Formic acid equivalent to HgCl.	Percentage of formic in volatile acid.
	cc.	*gm.*	*gm.*	*per cent*
29	23.6	0.6684	0.0652	60.0
124–2	29.5	0.8100	0.0790	59.0
S. lactis.	23.5	0.5854	0.0571	52.8

SUMMARY.

The fermentation products formed by this new group of pen-tose-fermenters from glucose, fructose, lactose, raffinose, and melezitose have been determined. All of these compounds are converted almost quantitatively into lactic acid. The lactic acid represents 90 per cent or more of the sugar fermented.

Glucose and fructose are almost entirely destroyed; in most cases, less than 0.1 gm. per 100 cc. of culture remained unfermented. Lactose, raffinose, and melezitose are less readily fermented; as much as 0.3 gm. per 100 cc. of culture may remain unfermented after 40 days. In 68 days all the sugar has been destroyed and a secondary fermentation of lactic acid begun. Volatile acid is formed in these old cultures.

Carbon dioxide is produced in small quantities, from 0.01 to 0.03 gm. per gm. of sugar destroyed. The amount is too small to represent a direct fermentation product. It is more plausible to regard it as a product of cell respiration. From these data it may be concluded that the bacteria produce no carboxylase.

The influence of a terminal alcohol group is manifested in the fermentation of mannitol. Ethyl alcohol, formic acid, and acetic acid are produced in addition to lactic acid. From 10 to 30 per cent of the total products is represented by these three compounds. It is suggested that these products are the result of correlative processes of reduction and oxidation.

The fermentation products of *Streptococcus lactis* like those of the pentose-fermenters are modified by the structural configuration of the fermented compound.

The pentose-fermenters produced only the inactive form of lactic acid while the active isomer was the form produced by the strain of *Streptococcus lactis*. Fractional crystallization failed to show any mixture of the two forms in any case.

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A STUDY OF THE ACETONE AND BUTYL ALCOHOL FERMENTATION OF VARIOUS CARBOHYDRATES.*

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A great deal of research has been performed on the acetone and butyl alcohol fermentation of maize, potatoes, horse-chestnuts, and other cereals rich in starch, in which various organisms of the widely distributed *Granulobacter* type have been employed. No systematic investigation has been made, however, of the action of this type of organism on other carbohydrates; namely, the various sugars, dextrins, and sugar alcohols. In consequence, at the suggestion of Professor Horace B. Speakman, a survey of these sources of carbon has been made with a freshly isolated soil organism. It was hoped that such an investigation would throw some light upon the biochemistry of the acetone and butyl alcohol fermentation by defining exactly which carbon compounds can be utilized by the organism.

In the early literature are found reports of work with various sugars but these are mainly concerned with the determination of end-products. Beijerinck (1) employed malt-wort in the butyl alcohol fermentation by means of *Granulobacter butylicum* and found maltose far better suited to the fermentation than glucose. Perdrix (2) submitted glucose, saccharose, and lactose to fermentation by an organism isolated from the Seine and called by him "*Bacillus amylozyme.*" The fermentation products from these sugars were acetic and butyric acids, hydrogen, and carbon dioxide, but no alcohols were formed. These latter products, however, were produced in considerable quantities from starchy

materials. Grimbert (3), on the other hand, in working with
Bacillus orthobutylicus, studied a large number of carbohydrates,
including glycerol, mannitol, glucose, invert sugar, saccharose,
maltose, lactose, arabinose, galactose, starch, dextrin, and inulin.
This investigator was interested mainly in the end-products of
the fermentation and did not study the course of the fermentation
in detail.   Later, amylaceous material was used extensively in the
manufacture of acetone and butyl alcohol by this fermentation
process (4, 5).   More recently, Speakman, and Reilly and co-
workers (6, 7, 8) have studied the biochemistry of the fermenta-
tion of starch by means of the Weizmann bacillus.   In none of
this later work has the fermentation of sugars been studied,
hence the present investigation was thought advisable.

In this work the following carbohydrates were studied:

Monosaccharides.	Disaccharides.	Trisaccharides.	Polysaccharides.
Xylose.	Sucrose.	Raffinose.	Starch.
Arabinose.	Maltose.	Melezitose.	Dextrin.
Glucose.	Lactose.		Inulin.
Fructose.	Melibiose.		
Mannose.	Trehalose.		
Galactose.			

In addition the two alcohols, glycerol and mannitol, were investi-
gated. A number of sugar mixtures also were submitted to
fermentation in order to study the behavior of the organism with
respect to sugar preference, if any existed, when both mono- and
disaccharides were offered. In these following experiments as in
previous work (6), the course of the fermentation was followed by
determinations of the titratable acidity at periodic intervals.
Sugar determinations were likewise made throughout the fer-
mentation period in order to observe the progress of carbohydrate
consumption and to ascertain, if possible, the exact method by
which each compound was utilized by the organism.

The specific organism employed in the fermentations was a
pure culture of a *Granulobacter* type of organism originally iso-
lated from a sample of fresh barley which was grown in central
Pennsylvania during the summer of 1919. When first isolated,
the organism gave sluggish fermentations. However, as a result

of constant cultivation on laboratory media for nearly 3 years, an exceedingly hardy strain of the bacillus has been produced. It was with this strain that the present fermentations were performed.

Bacteriological Methods.

Preparation of Media.—After experimenting with a large number of well known mineral salt solutions, the following mixture was adopted as being the best suited for the fermentations at hand:

Monopotassium phosphate.........................	1.00 gm.
Magnesium sulfate.................................	0.20 "
Sodium chloride...................................	0.01 "
Ferrous sulfate....................................	0.01 "
Bacto-peptone.....................................	5.00 "
Distilled water.................................... 1,000	cc.

In the first part of the investigation sugar concentrations of 5 per cent were used but later it was discovered that more complete fermentations resulted with 3 per cent solutions; therefore, approximately this concentration was used in the subsequent experiments. In preparing the medium, in the main, 750 cc. of the above nutrient solution were placed in a liter experimental flask. These flasks were specially made Pyrex Erlenmeyers provided with short outlet tubes attached about an inch from the bottom. During use the sampling outlets were fitted with short pieces of good rubber tubing closed tightly with close-fitting screw-clamps. The weighed amount of sugar or other carbohydrate was then added to the liquid and the flask shaken until complete solution was effected. Thereafter, two 12 cm. filter papers cut in 1 cm. strips, were added, the flask was plugged well with cotton and tied over with waxed paper to minimize evaporation during the incubation period. The addition of the strips of filter paper is understood when it is recalled that the organism used is essentially an anaerobe, and that the paper provides excellent centers for the formation of gas bubbles, which soon develop at such a rapid rate that very shortly all of the oxygen is driven out of the medium. At the height of the fermentation the paper is completely buoyed up to the surface of the medium in the form of a matted head, held together by the slime formed, and supported

by the gas bubbles. Under such anaerobic conditions extremely active fermentations occur. The flask of medium as prepared above is then sterilized in the autoclave at 5 pounds pressure for 30 or 40 minutes.

Inoculation and Incubation.—The organism used is an anaerobic spore former, the spores of which are heat-resistant. Spore stocks were kept on hand and active cultures were prepared from these in maize mash tubes. Cultures 24 hours old were employed throughout, and approximately the same proportion of culture was used for the various flasks. About 20 cc. of inoculum were used for a flask containing 750 cc. of medium. Tubes and flasks were both incubated at 37°C. which is the optimum temperature for fermentation. Before removing samples for analysis, the contents of the flasks were always shaken well in order to obtain even distribution of head and clear liquid.

Sampling.—Suitable samples were periodically removed through the side outlet tubes by opening the screw-clamps and allowing the well mixed solution to run into a graduate cylinder. Usually 50 cc. were taken which served for both acidity and sugar determinations. After closing the clamp tightly, the end of the rubber tube was carefully swabbed out with cotton soaked in saturated aqueous carbolic acid solution. The opening was then plugged with a fresh saturated swab. With this technique no contamination was encountered.

In other experiments in which the rarer sugars were used, smaller amounts of medium were prepared in ordinary Erlenmeyer flasks of various sizes. Samples were removed from these by means of sterilized pipettes.

Chemical Methods.

Acidity.—Duplicate 10 cc. samples of the fermenting medium were titrated directly with 0.1 N sodium hydroxide, using phenolphthalein as indicator.

Sugar Determinations.—Two 10 cc. portions of the original sample were pipetted into two 15 cc. centrifuge tubes and to each were added 1 cc. of saturated normal lead acetate solution and 2 cc. of alumina cream. After mixing well by inverting several times the tubes were whirled in a centrifuge for 5 minutes. Thereafter 10 cc. of the clear supernatant solution were pipetted from

each tube and transferred to a 100 cc. volumetric flask.   The sugar·
solution was then diluted to about 75 cc. and freed from lead by
adding small quantities of anhydrous potassium oxalate.   The
volume was then completed to the mark, the solution well mixed,
and finally filtered through a dry filter into a dry flask.   The
perfectly clear colorless solution was then used for the sugar
determinations.

Determination of Reducing Sugars.—Two methods were em-
ployed in the investigation for the estimation of reducing sugars.
The general method was that of Brown, Morris, and Millar (9),
which is a carefully standardized modification of Fehling's method.
Standard solutions were prepared as recommended by the authors
and frequent blanks on the reagents were run, due account thereof
being taken in all calculations.   The same standard apparatus
for heating, reducing, etc., was used throughout the investigation.
It was found that this gravimetric method yields excellent results
and, in truth, is much simpler to carry out than many of the more
recent involved volumetric methods.

The second method of determining reducing sugars was a
standardized modification of Barfoed's method, as recommended
by Legrand (10).   However, in it we employed a modified reagent
which minimizes the hydrolysis of disaccharides, instead of the
original Barfoed's solution.   This method was used to estimate
monosaccharides in the presence of reducing disaccharides such as
maltose, lactose, and melibiose; and it was found with careful
control to give fairly accurate results which were sufficiently
reliable for our purpose.   The new Barfoed reagent was prepared
as follows:

Neutral normal copper acetate...................... 50 gm.
Sodium acetate...................................... 50 "
Glacial acetic acid.................................. 5 cc.
Distilled water to................................... 1,000 "

By carefully standardizing the procedure good results were se-
cured with known mixtures, and it was found that under the
conditions noted below neither lactose nor maltose yielded any
cuprous oxide whatever with the test.   The procedure followed
was as given below:

A special reduction vessel was made from a smooth thick-walled
Pyrex Erlenmeyer flask of 150 cc. capacity by molding a lip on

one side of the neck so as to facilitate pouring and to serve as the steam outlet. A cover was made from a small thistle tube by cutting off and sealing the stem about ¾ of an inch from the bowl. When this was placed in the neck of the flask only a small aperture remained at the lip for the passage of steam and in this way excessive loss of acetic acid was prevented. The test for monosaccharides was performed with 25 cc. of sugar solution and 25 cc. of the reagent. An ordinary Bunsen burner was used for the heating, the flame being closely guarded against draughts by a shield. Before running the test with the sample the flame was carefully adjusted with a blank solution consisting of 25 cc. of Barfoed's solution and 25 cc. of water, so that the liquid reached the boiling point in exactly 3 minutes. With each test the time was noted and brisk boiling was allowed to proceed for exactly 3 minutes, at the end of which time the cuprous oxide was filtered off immediately and washed thoroughly with at least 300 cc. of boiling water. The crucible containing the oxide was next dried for 2 hours at 100°C. in a hot water oven, cooled for 20 minutes in a desiccator, and weighed.

It was found that glucose yielded approximately 1.61 times as much cuprous oxide with Fehling's solution as with Barfoed's. This factor, however, varies with the concentration of sugar, so its application is limited. However, where the factor has been used in the calculations it has been checked by determining the copper values by both methods of known solutions containing approximately the same concentrations of sugars as the samples.

<div align="center">EXPERIMENTAL.</div>

The carbohydrates employed were either Difco or Pfanstiehl preparations. Usually they were used as received without purification but in some cases recrystallization from alcohol of various strengths was resorted to in order to obtain purer products. Such was the case with glucose, maltose, and lactose.

<div align="center">*Fermentation of Monosaccharides.*</div>

In Table I are given the acidity and sugar data of the fermentation of these sugars. It is noted that a high initial acidity occurs in each case, due to the use of the particular acid medium which

contains dihydrogen phosphate. Examination of the table shows that these monosaccharides fall into two groups; *viz.*, Group I consists of glucose, fructose, and mannose; and Group II, xylose and galactose. The sugars of Group I are all fermented normally as evidenced by the rise in acidity to a maximum, followed by a decline and later by a second slight increase at the end of the fermentation. These changes are more clearly shown in Fig. 1. Here it is also seen that the sugars of Group II behave differently; the galactose curve never falls after once reaching the maximum point of 6.2 cc. In a somewhat similar manner the xylose acidity reaches a very high maximum and falls only a very

TABLE I.

Fermentation of Monosaccharides.

After inocula-tion.	0.1 N NaOH per 10 cc. of medium.					Sugar per 100 cc. of medium.				
	Glu-cose.	Fruc-tose.	Man-nose.	Galac-tose.	Xylose.	Glu-cose.	Fruc-tose.	Man-nose.	Galac-tose.	Xylose.
hrs.	cc.	cc.	cc.	cc.	cc.	gm.	gm.	gm.	gm.	gm.
0	1.30	1.12	1.28	0.91	1.00	3.82	3.54	2.76	2.88	2.55
6	1.90	2.56		2.02		3.81	3.39		2.77	
24	3.35	3.88	4.04	2.82	3.85	2.85	2.53	2.23	2.46	2.03
29	2.97	2.80	4.42	3.65	4.38	2.54		2.08		1.94
48	3.03	2.73	3.00	6.14	4.98	1.62	0.95	1.05	1.87	1.58
53	3.11		2.49	6.18	4.85	1.44		0.65		1.50
72	2.63	3.35	2.34	6.20	4.51	0.29	0.39	0.00	1.85	1.23
96	2.75		.			0.00	0.00			
120			2.23		3.87					
Sugar fermented, *per cent*						100	100	100	35	52

small amount, this occurring after the fermentation has practically run its course.

With respect to the percentages of sugar fermented there is likewise a marked difference in the action of the organism on these two groups of sugars. Whereas with glucose, fructose, and mannose the sugars are entirely removed from the medium, with the other two monosaccharides incomplete fermentations occur. About half of the xylose and only from 10 to 35 per cent of the galactose are utilized. In Fig. 2 the logarithms of the sugar concentrations at the different stages of the various fermentation periods are plotted, thus the curves indicate the different rates of

sugar consumption.   We see that in the first stage of the fermentation in each case, *i.e.* the stage of rapid acid formation during the
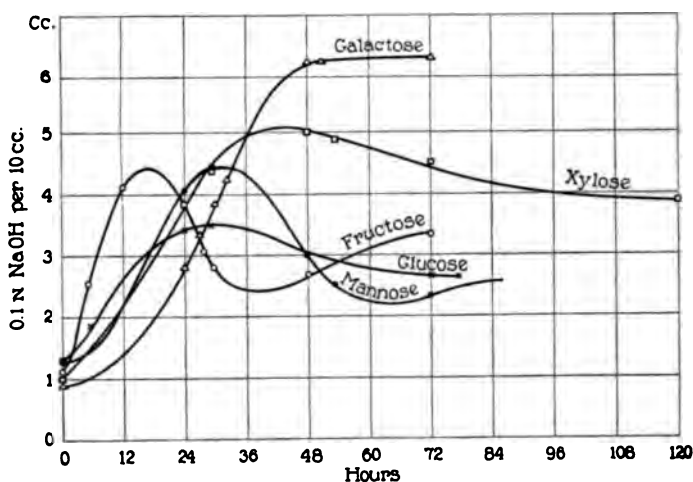
FIG. 1.   Acidity curves of the monosaccharide fermentations.
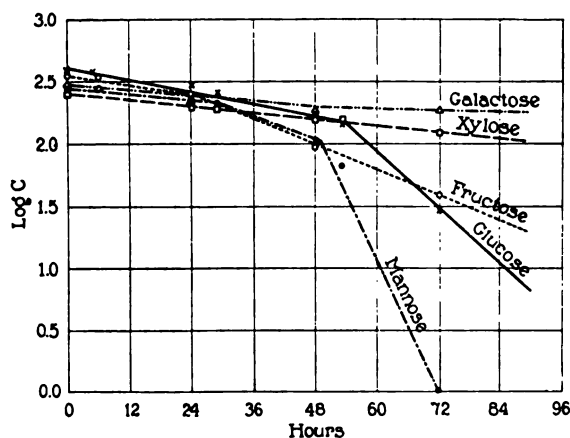
FIG. 2.   Sugar curves of the monosaccharide fermentations showing the different rates of sugar consumption.

first 30 or 36 hours, the rates of fermentation of the various sugars are practically identical.   Beyond this stage the curves show

striking differences and the sugars again fall into the same two groups. It is interesting to call to mind that these first three sugars, namely glucose, fructose, and mannose, are interconvertible into one another when in solution in the presence of a trace of alkali, through the formation of a common enolic form. Galactose, however, does not enter the enolic system and it is this sugar which does not ferment normally. The action of our organism on these particular sugars to a certain extent resembles that of ordinary yeast, except that xylose is partially fermented in the present case. In this connection it is interesting to point out the difference between the bacillus here studied and the lactobacillus of Peterson and Fred (11), which ferments glucose and galactose with equal ease but only consumes from 30 to 45 per cent of mannose even after 38 days incubation.

In addition to the above fermentations of monosaccharides, a few small ones were performed with the pentose sugar, arabinose, and with the methyl pentose, rhamnose. The former sugar is fermented in a similar manner to xylose. Rhamnose, however, is not attacked at all.

Fermentation of Disaccharides.

Sucrose, Maltose, and Lactose.—We shall first consider the commoner sugars, sucrose, maltose, and lactose, as they have been investigated in greater detail than the rarer disaccharides. The results of these fermentations are given in Table II, and it is readily seen that all of these sugars ferment normally with respect to acidity formation and sugar consumption. With sucrose, however, it is observed that all the sugar had not disappeared within the usual time. This is mainly due to the fact that a much more concentrated medium was employed, and also, owing to the initial presence of invert sugar, to a delay in the fermentation of the sucrose itself. These phenomena will be considered in more detail when the fermentation of mixtures of carbohydrates is discussed. Other sucrose experiments, in which 3 per cent sugar was used, have shown that this sugar is completely fermented but in a slightly longer time whenever an appreciable amount of invert sugar is present. Upon analyzing the fermented sucrose solution for sugar at the end of 144 hours fermentation, there was found only a trace of reducing material present after hydrolysis with

acid. All three of the common disaccharides are, therefore, completely fermented.

TABLE II.

Fermentation of Disaccharides.

After inoculation.	0.1 N NaOH per 10 cc. of medium.			Sugar per 100 cc. of medium.			
	Sucrose.	Maltose.	Lactose.	Sucrose.		Maltose.	Lactose.
				Invert sugar.	Sucrose.		
hrs.	cc.	cc.	cc.	gm.	gm.	gm	gm.
0	1.02	1.00	0.94	0.258	4.46	2.99	2.74
6	1.81	1.22	1.68	0.201	4.22	2.78	2.71
24	4.93	5.14	4.36	0.000	3.91	1.15	2.21
29	4.75	5.43	4.68		3.52	1.07	2.05
48	2.31	2.91	4.04		2.57	0.34	1.47
53	2.43	3.10			2.30	0.32	
72	2.96	3.19	2.92		1.46	0.00	0.51
77	3.08				1.31		
96			2.35		*		0.00
Sugar fermented, *per cent*............				100		100	100

* A trace of sucrose remained after 144 hours of fermentation.
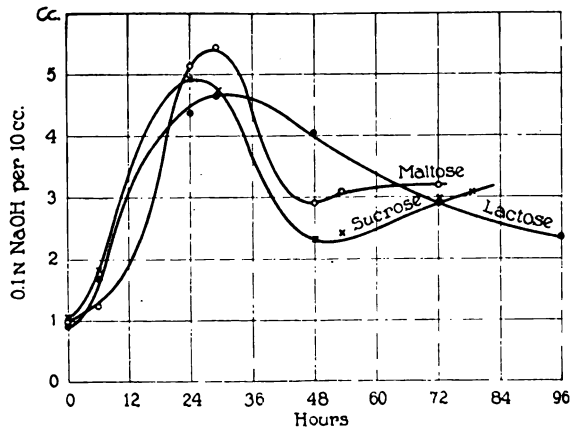
FIG. 3. Acidity curves of the disaccharide fermentations.

Fig. 3 shows the acidity changes which take place during the fermentation of these sugars. There is great similarity between the acidity curves of sucrose and maltose. Although the acidity

of the latter sugar reaches a higher maximum, the falls in each case are parallel and of approximately equal amounts. The lactose acidity curve, on the other hand, differs from these somewhat, it being more drawn out with a less rapid fall after the maximum is reached.

Passing now to the sugar curves as plotted in Fig. 4, we find that sucrose and lactose ferment at approximately the same rate, whereas maltose is attacked much more rapidly. Furthermore, starting with practically equal concentrations of maltose and lactose, the sugar in the former flask is completely consumed fully 24 hours sooner than that in the latter. With respect to the
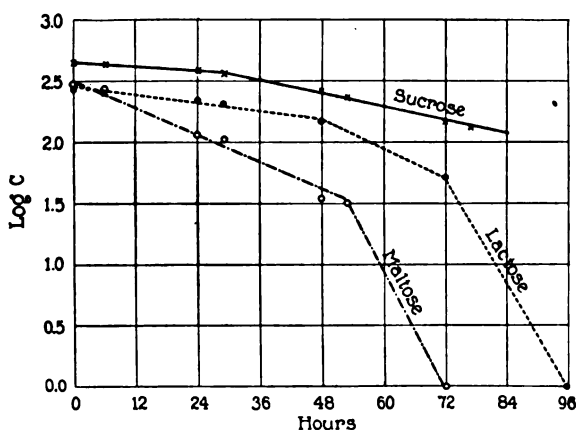
FIG. 4. Sugar curves of the disaccharide fermentations showing the different rates of sugar consumption.

fermentation of sucrose it should be mentioned that after the initial reducing sugar, present in the medium as a result of sterilization, had disappeared during the first few hours, at no time was any invert sugar observed during the whole fermentation period. Further consideration of this fact will be made later in this paper.

Melibiose and Trehalose.—In addition to the above common disaccharides small experiments were made with the two rare sugars, trehalose and melibiose. Trehalose failed to show the least sign of fermentation even after 2 weeks incubation, consequently it was concluded that the butyl bacillus is unable to split this sugar. Hence the organism does not contain trehalase.

This enzyme is contained, however, in certain fungi, especially *Aspergillus niger*, and many species of yeast, but according to Lafar no definite proof of its existence in bacteria has been brought forth. Fischer (12) contended that trehalase was identical with maltase but this is scarcely likely since the butyl organism here studied contains maltase but still does not hydrolyze trehalose.

A small quantity of melibiose was prepared according to the method of Bau (13), by hydrolyzing raffinose with 2 per cent acetic acid and fermenting out the fructose from the resulting mixture by means of a pure culture of top yeast. After purification with vegetable carbon, the remaining sugar solution, after adding the usual mineral salts and peptone, was inoculated with a butyl culture. For the first 10 hours active gassing occurred, but after this time the fermentation gradually subsided until at 24 hours no gas whatever was being evolved. The rise in acidity only amounted to 0.25 cc. of 0.1 N acid and the reducing sugars had only decreased a very small amount. The flask, however, was kept in the incubator for over a week but still no indication of a revived fermentation was observed, hence it was concluded that the initial gas production was due to the inoculum and to the destruction of the small amount of fructose left in the solution by the yeast. It is therefore concluded that melibiose alone is not fermented by the butyl bacillus.

Fermentation of Sugar Mixtures.

This series of experiments in which glucose was used in conjunction with other sugars was performed, first, for the purpose of finding out whether the butyl bacillus exhibited any preference for the simpler sugars, and secondly, for the purpose of determining whether in mixtures either sugar exerted any influence upon the fermentation of the other. The experiments were suggested by the peculiar behavior of the sucrose fermentation in the presence of an appreciable amount of invert sugar.

Glucose-Sucrose Mixture.—A sugar mixture consisting of 30 gm. of sucrose and 20 gm. per liter of medium was prepared and inoculated. The course of the fermentation was followed by the acidity readings and the consumption of the individual sugars was followed by periodic reducing sugar determinations both before and

after inversion. An active fermentation ensued immediately, the acidity attaining a maximum in about 30 hours. This was followed by the characteristic fall, and later by a marked rise which suggests a secondary fermentation. In Table III are given the results of this experiment as well as those of the maltose and lactose mixture fermentations which will be considered later. The sugar table of the glucose-sucrose experiment very clearly indicates that a

TABLE III.

Fermentation of Sugar Mixtures.

After inoculation.	0.1 N NaOH per 10 cc. of medium.			Sugar per 100 cc. of medium.					
	Glucose plus			Glucose plus					
				Sucrose.		Maltose.		Lactose.	
	Sucrose.	Maltose.	Lactose.	Glucose.	Sucrose.	Glucose.	Maltose.	Glucose.	Lactose.
hrs.	cc.	cc.	cc.	gm.	gm.	gm.	gm.	gm.	gm.
0	0.96	0.99	0.97	2.08	2.65	0.68	2.30	0.68	2.19
6		1.92	1.39					0.53	2.17
				(At 20 hrs.)					
12		4.18	3.44	1.70	2.65	0.21	1.59	0.25	
24	3.70	4.83	4.88	1.63	2.64	0.13	0.85	0.00	2.22*
				(At 44 hrs.)					
29	4.20	4.22	5.10	1.34	2.64	0.00	0.63		
48	3.13	2.96	4.16	1.24	2.54	0.00	0.25	0.00	1.59
68				0.37	2.59				
72	3.12	2.96	2.66	0.23	2.62	0.00	0.00	0.00	0.14
96			2.35						0.00
116	3.19			0.00	2.28				
120				0.00					
140	3.66			0.00	1.82				
214				0.00	Trace.				

* High value due to error in the determination.

marked preference is shown by the bacillus for glucose as compared with sucrose when the two sugars are present in the medium. We see that 72 hours were required for the removal of the glucose and that during that time no sucrose was utilized. After this occurred there was a gradual destruction of sucrose until it was all consumed. During this secondary fermentation there was no observable invert sugar present in the medium. This either

means that there is no exocellular sucrase or else that the invert
sugar, if formed, is destroyed as rapidly as it is produced. This
point will be considered later in the paper. The data from this
interesting experiment are plotted in Fig. 5 which shows very
clearly how the sucrose persists until after the glucose has dis-
appeared. Owing to lack of space the complete sucrose and
acidity curves are not shown, nevertheless the details are given
in the table. Normally the fermentation of glucose or sucrose
requires about 3 or 4 days but this double fermentation lasted for
fully 9 days.

Glucose-Maltose Mixture.—This fermentation was performed
similarly to the one just described except that a lower concentra-
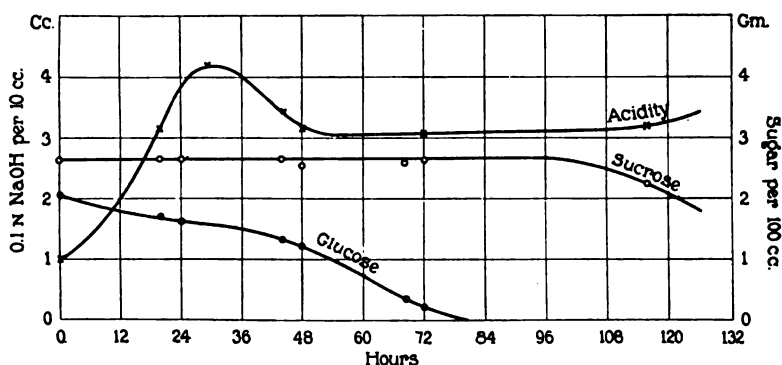
Fig. 5. Acidity and sugar curves of the fermentation of a glucose-
sucrose mixture.

tion of total sugar and a somewhat different ratio of glucose to
maltose were employed. The mixture consisted of 18 gm. of
recrystallized maltose and 4.5 gm. of purified glucose dissolved in
750 cc. of nutrient solution. After inoculation the course of the
fermentation was followed as before. However, in the present
case, since maltose is a reducing sugar, it became necessary to
employ Barfoed's method for the determination of the glucose in
the mixture.

The acidity, as shown in Table III, rises very rapidly and then
falls as in a typical pure maltose fermentation, differing in this
respect from the glucose-sucrose mixture which gave a curve
similar to that of a pure glucose fermentation. Also the sugar

data show that the amount of maltose starts to diminish at the very beginning as does also the glucose. However, since glucose is present in lower concentration it is consumed sooner than the maltose, but the rates of fermentation are approximately the same. Fig. 6 shows the sugar and acidity curves of the experiment. It is observed that these represent an entirely different type of fermentation than the one previously described. The organism does not show any preference for glucose or maltose. The question therefore arises: Does the butyl bacillus utilize the more complex maltose as such in the same way that it does the glucose, or is there an active maltase formed which immediately
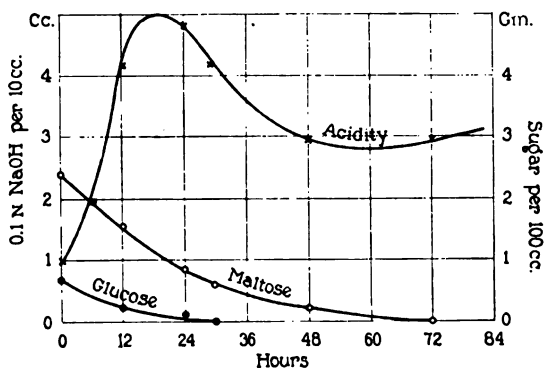
FIG. 6. Acidity and sugar curves of the fermentation of a glucose-maltose mixture.

attacks the maltose, hydrolyzing it into the simpler glucose which is then in turn fermented? This question will be considered later.

Glucose-Lactose Mixture.—A mixture of 4.5 gm. of pure glucose and 18 gm. of lactose was fermented in a manner similar to the two mixtures just considered. The data given in Table III indicate that this fermentation resembles the glucose-sucrose fermentation in that the disaccharide is not fermented until the last trace of glucose has disappeared. After this has taken place, the lactose is then gradually utilized until at 96 hours no more sugar remains in the medium. The short time in which the fermentation was completed was due in part to the lower concentration of sugars employed.

Glucose-Galactose Mixture.—Having observed in the experiments with the monosaccharides that glucose is completely fermented and that galactose is less than a third destroyed, and also in the lactose experiments that this sugar is completely fermented, quite likely after hydrolysis, it seemed possible that the presence of a readily fermented sugar, such a glucose might exert some accelerating influence upon the fermentation of galactose. If lactose is hydrolyzed it follows that this would result in furnishing the organism with a mixture of equal parts of glucose and galactose. Furthermore, since we find no trace of sugar at the end of the fermentation we must conclude that both these sugars are completely fermented under these conditions. From this it would seem that glucose does exert some beneficial influence upon the action of the organism on the difficultly fermentable galactose. In order to discover whether such is actually the case the following experiment was devised:

In each of three experimental flasks were placed 750 cc. of nutrient solution and to the first were added 20 gm. of glucose, to the second 20 gm. of galactose, and to the third 10 gm. of each of these two sugars. After sterilization the flasks were inoculated with similar cultures. Acidity readings were made at regular intervals during the fermentations but sugar determinations were made only at the start and at the finish. The data of these experiments are given in Table IV. The glucose and galactose fermentations were of the same type as previously described, that is, the acidity of the former rose to a maximum and then fell while that of the latter maintained its high value throughout the course of the fermentation. However, the data of the mixed fermentation show interesting differences. In the first place the acidity is seen to rise sharply until a high value is reached, which is followed by a fall as in a pure glucose fermentation. Later a second rise is noted until a second high value is attained, which is considerably higher than the first. This acidity is then maintained as in a pure galactose fermentation. Considering only the acidity curves, which are plotted in Fig. 7, one can see that the mixed fermentation consists of two separate and distinct fermentations, one displaying the characteristics of a pure glucose fermentation and the other exhibiting those of a pure galactose fermentation. The first fermentation is finished in the first 36

hours and the second is practically complete in a further similar period.

<div align="center">TABLE IV.</div>

Fermentation of Glucose, Galactose, and a Glucose-Galactose Mixture.

After inoculation.	0.1 N NaOH per 10 cc. of medium.			Sugar per 100 cc. of medium.		
	Glucose.	Galactose.	Ratio 1:1. Glucose-galactose.	Glucose.	Galactose.	Ratio 1:1. Glucose-galactose.
hrs.	*cc.*	*cc.*	*cc.*	*gm.*	*gm.*	*gm.*
0	1.01	1.02	0.95	2.54	2.08	2.31
6	1.59	1.59	1.82			
11	3.45	3.36	3.72			
24	4.12	5.19	4.18			
30	3.74	5.48	3.54			
48	2.81	5.92	3.88			
72	2.53	6.02	4.92			
96	2.55	6.04	4.91	0.00	1.97	0.55
Sugar fermented, *per cent*..........				100	10	{ Glucose ...100 { Galactose.. 47
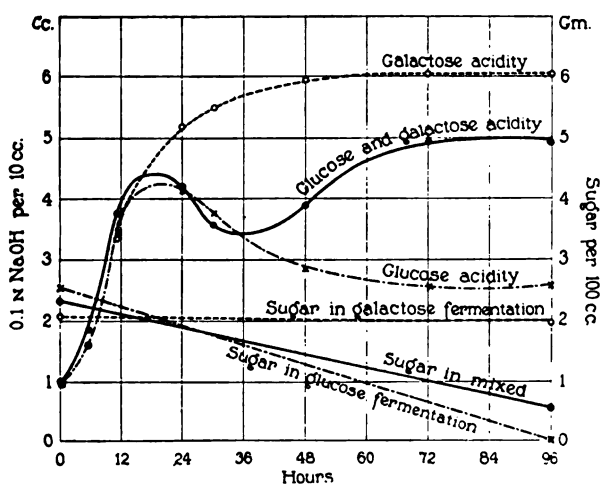
FIG. 7. Curves showing the comparison between the pure glucose, the pure galactose, and the mixed glucose-galactose fermentations.

We find that the total reducing sugar value has fallen from an initial concentration of 2.31 gm. per 100 cc. to a concentration of

0.55 gm. per 100 cc. (calculated as galactose). This shows a total loss of sugar of about 76 per cent. However, upon testing the resulting beer of the fermentation with the osazone test, using phenylhydrazine hydrochloride and sodium acetate, no glucosazone was detected, but large quantities of galactosazone were obtained. Assuming, therefore, that all of the glucose had disappeared from the medium, we find that a little over half of the galactose was fermented whereas with the pure galactose fermentation only about 10 per cent was consumed.

Fermentation of Trisaccharides.

Raffinose.—The trisaccharide, raffinose, is completely fermented by bottom yeast owing to the presence of both invertase and melibiase. Top yeast, on the other hand, only partially ferments it, leaving as a residue the disaccharide, melibiose, because of the fact that these particular types of yeast do not contain the enzyme, melibiase. A number of molds and bacteria also ferment this trisaccharide, either partially or completely.

The medium for the raffinose fermentation was prepared by dissolving 9 gm. of the sugar in 350 cc. of nutrient solution and sterilizing at 5 pounds pressure for half an hour. After inoculation periodic samples were withdrawn by means of sterilized pipettes. These portions were analyzed for acidity, monosaccharides, and disaccharides. Use was made of both the Barfoed and the Brown, Morris, and Millar methods. By studying the acidity data given in Table V, it is observed that an abnormal fermentation takes place which is almost identical with that of the galactose experiments. The initial rise in acidity, however, is much slower in the case of raffinose and it reaches a lower maximum. Fig. 8 shows the curve based on these acidity data.

We will now consider the possible ways in which this trisaccharide can be attacked by any fermenting mechanism. Raffinose is composed of three monosaccharide molecules; namely, glucose, fructose, and galactose. Different enzymes have different actions upon the sugar, splitting it in different ways. There are, therefore, four possible ways by which raffinose can be attacked: namely, fermented (1) directly without preliminary hydrolysis; (2) after complete hydrolysis by the enzyme, raffinase;

(3) after the complete hydrolysis by the two enzymes emulsin and sucrase; and (4) after partial hydrolysis by sucrase, yielding

TABLE V.

Acidities of the Raffinose, Dextrin, and Starch Fermentations.

| After inoculation. | 0.1 N NaOH per 10 cc. of medium. | | | |
| | Raffinose. | Dextrin. | | Starch. |
		Acid hydrolyzed.	Amylase hydrolyzed.	
hrs.	*cc.*	*cc.*	*cc.*	*cc.*
0	0.91	1.02	1.09	0.91
6	1.53	1.40	1.68	1.78
24	3.02	4.38	4.57	4.54
29	3.32	4.77	4.93	4.50
48	4.17	5.01	4.32	3.23
53	4.38	4.96	4.24	3.09
72	4.58	5.16	3.74	3.20
96		5.08	3.47	
120	4.62			

TABLE VI.

Sugar Data of the Raffinose, Dextrin, and Starch Fermentations.

| After inoculation. | Sugar per 100 cc. of medium. | | | | |
| | Raffinose. | | Dextrin. | | Starch. |
	Fructose.	Melibiose.	Acid hydrolyzed.	Amylase hydrolyzed.	
hrs.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
0	0.00	0.00	0.00	0.078	0.00
6	0.00	0.00	0.091	0.063	0.18
24		0.141	0.208	0.312	0.66
29	0.294				0.71
48	0.361	0.368	0.104	0.156	0.74
53	0.406	0.356			0.72
72	0.450	0.382	0.109	0.052	0.47
84					0.25

fructose and melibiose. Of these four possible methods of attack, the first would appear to be highly improbable when we consider the method by which the disaccharides are utilized. The data

given in Table VII eliminates the second possible method because the presence of a disaccharide is clearly indicated by the wide differences between the successive Barfoed and Fehling values of the fermenting solution.   To confirm this a mixture of equal proportions of glucose, fructose, and galactose, which by the way would result in case raffinose was completely hydrolyzed by raffinase, was tested for reducing values by both methods and in no case gave such wide differences.   It is, therefore, quite evident that some sugar is formed during the fermentation which reduces Fehling's solution and does not reduce Barfoed's.   This fact also eliminates Method 3, otherwise sucrose, a non-reducing sugar,
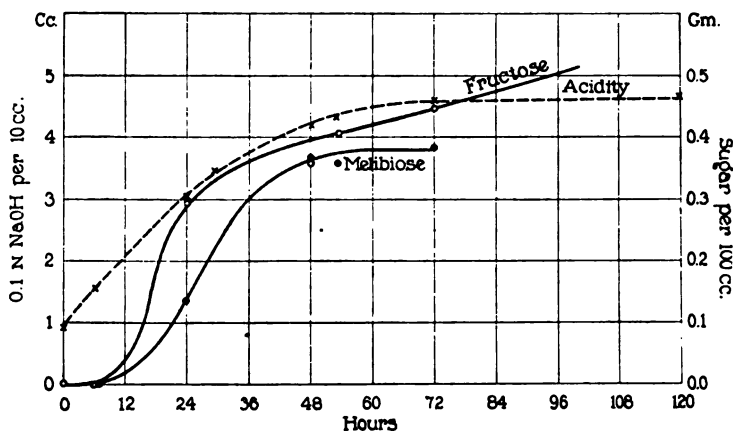
Fig. 8.   Acidity and sugar curves of the raffinose fermentation.

would first form and then be split up by sucrase which is known to be present.   It follows, therefore, that the butyl organism studied attacks raffinose by Method 4; that is, by splitting it into fructose and melibiose by means of sucrase.

In order to confirm this fact the osazone test was again resorted to for demonstration of the presence of melibiose and fructose in the fermented residue.   Two osazones were prepared which after fractionation and repeated recrystallization from boiling water and 50 per cent alcohol, proved to be those of melibiose and fructose.   The melibiosazone crystallized in fine, delicate medallions, having sharp saber-like points radiating from the center.   These crystals melted from 180–182°C. which agrees closely to Browne's

(14) figure of 179° for melibiosazone. The other compound resembled the osazone of glucose, fructose, and mannose, in structure, and melted at 205°C. It is no doubt formed from fructose since this sugar is the one produced from raffinose when melibiose is split off.

Since fructose and melibiose are present in the fermented beer it follows that these sugars are formed during the fermentation by the action of the enzyme, sucrase, which is present in the butyl bacillus. This enzyme is not secreted, however, because in the presence of toluene no reducing sugars are produced in a pure raffinose medium inoculated with a large volume of active culture. Hence the enzyme must function within the cell. In view of this fact it is supposed, in considering the mechanism of the raffinose fermentation, that the sugar enters the cell and is there hydrolyzed

TABLE VII.

Raffinose Fermentation.

After inoculation.	Cuprous oxide per 25 cc. of solution.		
	Barfoed's value.	Fehling's equivalent.	Fehling's value.
hrs.	*mg.*	*mg.*	*mg.*
29	14.8	23.8	
48	18.0	29.0	47.5
53	20.1	32.4	50.3
72	22.3	35.9	55.1

into fructose and melibiose. Fructose, being readily fermented is at first consumed, but melibiose not being so easily fermented accumulates after a short time in the cell. It is due to the active destruction of fructose that vigorous gassing and a rise in acidity occur. After a certain amount of melibiose has accumulated in the cell it diffuses out into the medium and is there detected by Fehling's solution. Eventually after about 24 hours some unknown inhibiting factor or combination of factors begins to interfere and the fermentation is retarded. This is accompanied by the slowing up of gas production and a slackening up of the acid formation. Beyond this point fructose begins to accumulate and, consequently, diffuses from the cell into the medium where its presence is detected by the Barfoed test. Finally the inhibiting influence becomes so effective that the fermentation stops alto-

gether, the acidity ceases to rise and gas evolution ends. As a result no more fructose or melibiose are produced on account of the stoppage of the internal hydrolyzing processes of the cell. Hence the values of these sugars remain constant in the medium as shown by Table VI.

Melezitose.—Melezitose, the other trisaccharide studied in addition to raffinose, is not fermented by yeast but it has been shown that the mold, *Aspergillus niger,* slowly hydrolyzes it to glucose and turanose without subsequent fermentation at 50°C. On account of these peculiar facts it was thought that it would be interesting to study the action of the butyl bacillus on this sugar.

A small flask of medium, containing 3 gm. of the sugar, was prepared and inoculated with an active culture. Acidity determinations were made periodically but only initial and final sugar values were obtained.   The acidity data are found in Table VIII and the curve of the same is given in Fig. 10. We see that the melezitose fermentation is similar to the one just considered in detail, particularly with respect to acidity changes. There is no drop once the maximum is reached; thus an incomplete fermentation is indicated. This fact was confirmed by the sugar determinations. These were performed after complete hydrolysis with hydrochloric acid. At the beginning of the fermentation the medium contained 3 gm. of melezitose per 100 cc., and at the end after 120 hours, there remained 1.64 gm., showing that 1.36 gm. or 45 per cent had been consumed.   It is interesting to note that during the entire fermentation period no reducing sugar was observed in the medium, from which we conclude that the simpler sugars resulting from the hydrolysis of the trisaccharide were consumed as rapidly as formed.

Fermentation of Polysaccharides.

Starch.—A 5 per cent mash of potato starch in nutrient solution was employed and the acidity and reducing sugar changes were followed periodically throughout the course of the fermentation. The acidity data as given in Table V show that the fermentation was normal. These data are plotted in Fig. 9. At the time of inoculation the starch mash was extremely gelatinous and free from reducing sugar, but very soon after inoculation liquefaction commenced and sugar formation began. The sugar formed

has been identified as glucose by the osazone reaction (6). The concentration of sugar gradually increased until a maximum was attained at 36 hours, as shown in Table VI and Fig. 9. Immediately after this a sharp fall in the sugar content was observed which continued throughout the remainder of the fermentation period until at the end no more sugar remained.

Dextrins.—Two dextrins were prepared, one by the acid hydrolysis of potato starch and the other by malt amylase hydrolysis of the same material. The two preparations were purified by re-
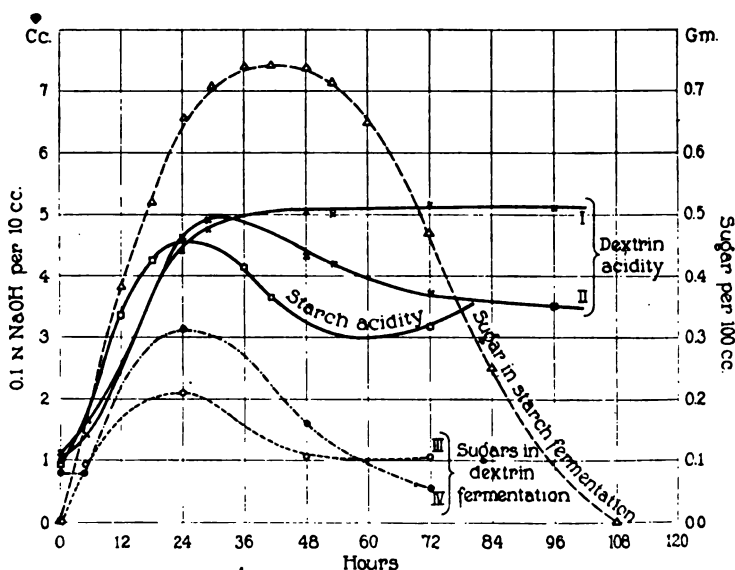
FIG. 9. Acidity and sugar curves of the starch and the dextrin fermentations.

peated precipitations with alcohol, and with one intermediate fermentation by yeast to remove occluded sugar. Finally two stable products were obtained which had only a slight reducing power when boiled with Fehling's solution. The acid-hydrolyzed product consisted chiefly of erythrodextrin, while the amylase product was mainly achroodextrin.

These two dextrins were then used in the following experiment. Two flasks of media were prepared by dissolving 9 gm. of each dextrin in separate 350 cc. portions of nutrient solution. After

inoculation with 10 cc. of active culture the acidity and reducing values of each fermenting mixture were followed periodically. The acidity data are given in Table V. The figures show that the two fermentations are not at all alike. With the biological product a perfectly normal fermentation occurs, but in the case of the acid-hydrolyzed dextrin the fermentation is quite abnormal, comparing well with that of galactose. These data are plotted in Fig. 9 as Curves I and II, of which Curve I represents the acidity changes occurring in the fermentation of the acid product, and Curve II those of the amylase product. The former curve is of the same type as the galactose acidity curve while the latter resembles the curve of a lactose fermentation. The reducing sugar data of both fermentations are found in Table VI and are plotted in Fig. 9 as Curves I and II. These two curves are similar to the sugar curve of the starch fermentation. In order to determine the extent to which each type of dextrin was consumed, samples of the fermented solution were filtered and aliquots analyzed for residual dextrin by completely hydrolyzing with hydrochloric acid and determining the glucose formed. It was found that with the acid product 3.26 gm. of dextrin remained in the solution indicating a consumption of about 60 per cent. The amylase product was completely fermented. This seems to indicate a marked difference in the character of the two dextrins prepared by different methods, one chemical and the other biological. In this connection it is interesting to draw attention to the fact that dextrins prepared by the action of malt diastase are much more easily hydrolyzed by pancreatic juice than are dextrins which are prepared by the acid hydrolysis of starch (15). However, in the present case, until further evidence is secured by additional experiments, it cannot be definitely stated that the difference between the availabilities of the two dextrins here studied is entirely due to the modes of preparation or to the fact that in the one case erythrodextrin was used and in the other the achroodextrin was employed. Further study will be undertaken in the near future on this point as it appears to be of more than passing interest.

Inulin.—A small flask of inulin was fermented and the course was followed by the acidity determinations. Initial and final inulin values were also determined so as to discover to what extent

the carbohydrate was consumed. The results of the acidity titrations are given in Table VIII and the curve of the same is found in Fig. 10. In this case also an incomplete fermentation is encountered, as shown by the maintained high acidity readings. This fact was likewise borne out by the inulin

TABLE VIII.

Acidities of the Melezitose, Inulin, and Mannitol Fermentations.

After inoculation.	0.1 N NaOH per 10 cc. of medium.		
	Melezitose.	Inulin.	Mannitol.
hrs.	*cc.*	*cc.*	*cc.*
0	1.01	0.96	1.02
24	4.18	3.47	3.36
30	4.41	4.23	4.02
48	5.05	4.36	4.83
54	5.00	4 38	4.93
72	5.05	4.47	5.05
120	5.06	4.47	4.86
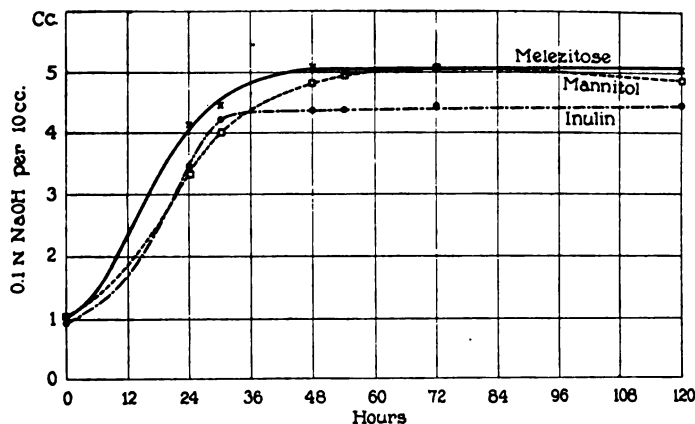
Fig. 10. Acidity curves of the melezitose, inulin, and mannitol fermentations.

determinations, performed by first hydrolyzing the solutions with hydrochloric acid and then determining the fructose formed. Slightly less than half of the inulin was consumed by the bacillus. During the fermentation period no reducing sugar appeared in the medium until near the end of the fermentation.

Fermentation of Alcohols.

Mannitol.—The medium used in this experiment consisted of a 3 per cent pure mannitol solution with the usual nutrient salts added in the correct proportions. Upon inoculation this medium was found to ferment vigorously with extremely active gas production and a rapid rise in acidity. This fermentation proved to be one of the abnormal type as shown by the maintained high acid value after the maximum was reached. The acidity data are plotted in Fig. 10. No data was obtained showing the extent to which this carbohydrate was fermented by the organism.

Glycerol.—A medium containing 3 per cent of pure glycerol was prepared and inoculated with active culture. After the carbohydrate contained in the added inoculum was consumed there was no evidence whatever of any fermentation of the glycerol. Hence it is concluded that this material is not available as a source of carbon for the butyl bacillus.

Hydrolysis of Carbohydrates in the Presence of Toluene.

. Knowing from previous experiments that the organism is capable of utilizing such carbohydrates as sucrose, maltose, lactose, raffinose, melezitose, dextrin, starch, and inulin, as well as the simpler monosaccharides, the question at once arose as to the exact manner in which these compounds were attacked. In the case of colloidal substances such as starch, dextrin, and inulin, it is obvious that the organism must first effect hydrolysis before these materials can enter the cell. Hence it follows that the bacillus must secrete exocellular enzymes to effect these hydrolyses. Sugars, however, are capable of passing through the membrane with facility, and initial hydrolysis is not therefore essential. For each sugar there are three possible methods of attack; *viz.*, (1) direct utilization without preliminary hydrolysis; (2) preliminary hydrolysis by an exocellular enzyme; (3) hydrolysis within the cell by an enzyme not secreted. If the first of these methods obtains we must assume the presence within the cell of an elaborate enzyme system capable of converting a large number of sugars of very different structure into common end-products. On the other hand, it seems more probable that the di- and trisaccharides are first split into their respective simple hexoses either without

or within the cell, and that these sugars are then attacked by the acid-forming enzymes. The raffinose experiments have demonstrated in a definite manner the existence of sucrase. Assuming that lactase and maltase also exist, our conception of the enzyme system becomes much simpler. Any sugar, for instance, which, following hydrolysis, yields the hexoses, glucose, fructose, or mannose, can be fermented by the same enzyme system owing to the fact that these sugars are interchangeable, passing through a common enolic form when in solution. Galactose, however, when produced by hydrolysis, requires a special mode of attack, since this hexose does not pass into the same enolic form as do the other three sugars mentioned. Nevertheless, this special galacto-enzyme, which is required, exists in the butyl organism because we find that pure galactose is at least partially fermented and lactose completely.

Since there is little doubt but that the complex sugars are first hydrolyzed before they are fermented, the question still remains as to whether these hydrolyses take place outside or inside the cell. The problem is considered in the following series of experiments. The method of study, briefly stated, consisted of preparing flasks of the various carbohydrates; inoculating these with active cultures; incubating for about 24 hours; and finally adding toluene to stop further cell activity. Changes in the reducing sugar values were then noted and compared with suitable controls in order to determine whether any hydrolytic action had taken place by secreted enzymes.

Sucrose, Maltose, and Lactose.—To 300 cc. flasks, each containing 200 cc. of nutrient solution, were added 6 gm. of the sugar to be tested. The solutions were sterilized at 5 pounds pressure for $\frac{1}{4}$ hour. No hydrolysis occurred in the sucrose flask and only a slight amount in those containing maltose and lactose. Each flask was inoculated with 10 cc. of active culture and incubated at 37°C. for 26 hours. To 100 cc. portions of the actively fermenting cultures placed in 150 cc. sterile flasks, were added 15 cc. of toluene, and after shaking, these solutions were analyzed for monosaccharides by our modified Barfoed method. The residues in the original flasks were boiled and analyzed in the same manner. All six flasks were then incubated. After $27\frac{1}{2}$ and 99 hours the incubated solutions were again examined for monosaccharides.

This same experiment was repeated as a check on the results. The two sets of data are given in Table IX.

It is clearly shown that maltase is secreted by the butyl organism. On the other hand, there is no evidence of any secretion of sucrase or lactase. Therefore, maltose alone is hydrolyzed in part at least outside of the cell. This seems to be in keeping with our previous observation that in a mixture of glucose and maltose, the latter sugar is fermented simultaneously with the glucose. We find now that this is due to the immediate secretion of maltase which at once begins to attack the disaccharide present in the

TABLE IX.

Toluene Experiments.

After toluene addition.	Cuprous oxide per 25 cc. of solution.		
	Sucrose.	Maltose.	Lactose.
Series I.			
hrs.	*mg.*	*mg.*	*mg.*
0	0.0	5.1	0.5
27½	0.0	31.5	0.0
99	0.0	57.3	0.0
Series II.			
0	0.0	0.0	0.0
48	0.0	43.4	0.0
Boiled controls after 48 hrs......	0.0	Trace.	0.0

medium. In the case of mixtures containing either sucrose or lactose no suitable enzymes are secreted and we find that the disaccharides are not molested until all of the glucose has disappeared. What is the reason for this selection? Is it due to differences in the relative penetration of di- and monosaccharides through the cell membrane or is it due to a natural preference of the organism for the simpler and more easily oxidized food? At present we are unable to answer these questions.

Raffinose.—A raffinose medium, containing 3 per cent of the sugar, was inoculated with active culture and incubated for 20 hours after which toluene was added. No reducing sugars were

present. The flask was replaced in the incubator and allowed to remain for 72 hours. Upon examination with Fehling's solution no reducing sugar was found. This confirmed the previous observation that sucrase is not secreted.

Starch, Dextrin, and Inulin.—With regard to the polysaccharides, starch, dextrin, and inulin, experiments of a similar kind were performed. The changes taking place in the flasks to which toluene had been added and in the boiled controls were followed by means of sugar determinations and iodine color reactions. The results obtained show that in all three cases hydrolysis is effected by means of secreted enzymes. We conclude, therefore, that the two enzymes, amylase and inulinase, are secreted by the bacillus.

SUMMARY.

1. The fermentations obtained with the various carbohydrates used in this investigation are of two types. The first type, to be regarded as the normal, is characterized by a decided fall in the acidity after the maximum is reached, and also by the complete consumption of the carbohydrate. The group of abnormal fermentations is characterized by the persistence of a high acidity and also by the incomplete destruction of the carbohydrate.

2. Glucose, fructose, mannose, sucrose, lactose, and starch belong to Group I, while galactose, xylose, arabinose, raffinose, melezitose, inulin, and mannitol constitute Group II. Dextrin belongs to either group depending upon the method used in the preparation of the sample fermented. The biological product is completely fermented, but the dextrin prepared by the acid hydrolysis of starch is only partially consumed.

3. Trehalose, rhamnose, melibiose, and glycerol are not fermented.

4. The butyl organism secretes the following enzymes: amylase, inulinase, and maltase; but it does not secrete sucrase, lactase, or raffinase.

5. Raffinose is hydrolyzed within the cell by sucrase into melibiose and fructose.

6. The organism first completely removes the hexoses, with the exception of galactose, from mixtures also containing sucrose and lactose. Maltose on the other hand is fermented concurrently with glucose, fructose, or mannose.

I wish to acknowledge with pleasure my indebtedness to Professor Horace B. Speakman for his ever ready counsel and advice throughout this work.

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ANIMAL CALORIMETRY.

THE PRODUCTION OF FAT FROM PROTEIN.

BY H. V. ATKINSON, DAVID RAPPORT, AND GRAHAM LUSK.

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New York City.)*

In the second paper of this series Williams, Riche, and Lusk
(1) described experiments which showed the hourly metabolism
of a dog after giving 1,200 gm. of meat. The dog weighed 13.5
kilos and was maintained by administering 1,200 and 700 gm. of
meat on alternate days. When given the larger quantity of meat,
which contained about 1,200 calories, the heat production was
about 800 calories daily, the dog resting quietly. The protein
content of the meat was the equivalent of 900 calories. It follows
that the protein ingested was alone more than sufficient for the
dog's needs. However, when 700 gm. of meat were given the
heat production of the resting quiet dog was about 700 calories
daily, the ingested meat contained the same number of calories,
while the protein element contained only 525 calories. Under
these latter conditions one would not expect any permanent re-
tention of glycogen derived from the protein metabolism of this
dog during a day of ordinary activity following the administra-
tion of 700 gm. of meat.

The conditions were therefore favorable for the deposit of
glycogen in the liver following the administration of 1,200 gm. of
meat. As a matter of fact, during a period of 14 hours after the
administration of this large amount of meat there was much
less carbon eliminated in the respiration than corresponded to the
protein metabolism of the time as measured by the excretion of

nitrogen in the urine. That this carbon was retained in the form of glycogen was proved by the fact that the quantity of oxygen absorbed agreed with this hypothesis, and that the heat calculated on the basis of a retention of glycogen agreed with the heat as measured by the calorimeter.

The Work of Atkinson and Lusk.

Fat Formation.—Atkinson and Lusk (2) performed a long series of experiments with the object of discovering the· quality of the material retained when, after giving meat in large quantity to a dog, the amount of protein metabolism was in excess of the fuel needed for the heat production of the time.

A résumé of these experiments is given in Table I.

It will be remembered that when the protein of meat is oxidized in the body the respiratory quotient is 0.801.

It is evident from the table that in ten out of twelve experiments, after giving meat amounting to between 700 and 1,300 gm. daily, the respiratory quotients varied between 0.787 and 0.808, as appears below:

Experiment No..	31	48	54	51	47	46	55	30	34	32
R. Q.	0.787	0.793	0.794	0.795	0.795	0.796	0.797	0.800	0.800	0.808
R. Q. of deposit..	0.960	0.860	0.860		0.840		0.830	0.830	0.830	0.770

In Experiments 51 and 46 there was no retention of protein carbon, whereas in six of the experiments the retained carbon was held in such a form that, had it been oxidized, it would have yielded respiratory quotients of between 0.83 and 0.86, which indicates the retention of a pabulum containing only about half of its calories in fat and half in carbohydrate. By weight this would indicate the retention of approximately 1 gm. of fat to every 2 gm. of glycogen.

Only after the ingestion of meat in very large quantities— 1,100 and 1,300 gm.—was there evidence of the conversion of protein into fat as the dominant feature of the process. This appears below:

Experiment No.	33a	33b	56
R. Q.	0.831	0.843	0.826
R. Q. of deposit.	0.680	0.490	0.710

As the respiratory quotient of fat is 0.707, the above results warranted the conclusion that in the case of excessive ingestion of meat by a dog the retained pabulum might be laid down as fat when the circumstances were favorable. It was noted that it was very difficult to induce the dog to take meat in these very large quantities.

During the period of experimentation twelve alcohol checks were made. The average of all the respiratory quotients was 0.668 (theory 0.667) and the heat recovered was 0.3 per cent greater then the heat calculated to be obtainable from the combustion of the alcohol.

The extent of the hourly retention of carbon calculated from the protein metabolism (measured by the urinary nitrogen) and the CO_2 output of the period bear no relation to the total heat production, as appears in the following tabulation:

Experiment No..	55	47	34	30	48	54	56	33a	31	32
Protein C retained, *gm*......	0.91	0.77	0.70	0.66	0.61	0.59	0.59	0.56	0.48	0.32
Calories of metabolism.......	32.00	29.90	34.00	31.50	29.40	31.10	31.60	31.70	34.30	34.90

It is well known that the deposition of glycogen does not increase the heat production. In Experiments 33a and 56, in which fat appeared to be the sole material deposited, the heat production was no higher than in Experiments 55, 47, 30, 48, 54, and 31, when fat and glycogen were laid down in about equal caloric equivalents.

It is therefore apparent that *the specific dynamic action of protein is not due to the formation or deposition of synthetically formed glycogen or fat.*

Basal Metabolism and "Deposit Protein."—"Deposit protein" is that quantity of protein which is held in the body after excessive protein ingestion, and which is gradually eliminated from the cells in which it is stored upon the cessation of a high protein diet. Historically it is the same as Voit's "circulating protein." Benedict, Miles, Roth, and Smith (3) were the first to associate the loss of "deposit protein"—which they called "surplus cellular nitrogen"—with a reduction in the total metabolism of men.

TABLE I.

Effect of Meat Ingestion on Hourly Metabolism. Series I. Dog XVIII.

Experiment No.	Date.	Food.	Number of hours.	Urine N.	R. Q.	Calories. Indirect.	Calories. Direct.	C deposited.	R. Q. of deposited material.	Hours after food.
	1919			*gm.*				*gm.*		
27	Feb. 6	Basal.	2	0.15	0.840	15.92	16.08			(Weight = 11.24 kg.)
30	" 18	Meat, 800 gm.	3	1.46	0.800	31.47	32.75	+0.6?	0.83	4, 5, 6
31	" 19	" 900 "	3	1.47	0.787	34.33	34.14	+0.48	0.96	4, 5, 6
32	" 20	" 1,000 "	4	1.46	0.808	34.90	35.87	+0.32	0.77	4, 5, 6, 7
33	" 21	" 1,100 "	2a	1.45	0.831	31.65	31.36	+0.56	0.68	4, 5
			2b	1.45	0.843	35.28	34.54	+0.25	0.49	6, 7
	Feb. 22	Meat, 700 gm.								
	" 23	" 700 "								
34	" 24	" 1,080 " *	2	1.57	0.800	34.00	34.12	+0.70	0.83	4, 5
35	" 26	Basal.	3	0.27	0.820	19.74	19.59			(Weight = 12.07 kg.)
36	" 27	"	3	0.20	0.830	18.25	17.16			
37	" 28	"	2	0.17	0.850	17.30	16.95			
38	Mar. 1	"	2	0.15	0.820	18.21	(18.21)			
39	" 3	"	3	0.15	0.850	17.57	17.22			
43	" 12	"	2	0.15	0.810	17.08	16.99			(Weight = 11.50 kg.)

46	Mar. 17	Meat, 1,200 gm.	3	1.02	0.796	26.57	28.10	0		5, 6, 7 after 1 day's fast.
47	" 18	" 800 "	3	1.44	0.795	29.90	30.77	+0.77	0.84	5, 6, 7
48	" 19	" 800 "	4	1.35	0.793	29.37	30.27	+0.61	0.86	5 to 8
49	" 22	Basal.	2	0.23	0.790	17.72	17.54			
50	" 24	"	2	0.16	0.840	17.26	16.87			
51	" 28	Meat, 800 gm.	4	1.02	0.795	27.04	27.52	+0.59	0.86	5 to 8 after 4 days' fast.
54	Apr. 15	" 800 "	4	1.41	0.794	31.07	30.57	+0.91	0.83	5 to 8
55	" 16	" 1,000 "	4	1.58	0.797	31.97	31.98	+0.59	0.71	5 to 8
56	" 19	" 1,300 "	4	1.47	0.826	31.62	33.25	+0.59		5 to 8 after 1 day's fast.
Total						568.22	571.85			

* Standard diet at 5 p.m. and thereafter daily until Mar. 15.

TABLE—III *The Effect of Meat I...*

Date.	Experiment No.	Time	CO$_2$	O$_2$	R.Q.	H$_2$O	Urine N.	Non-protein.			Pro-
								CO$_2$	O$_2$	R.Q.	tein.
1920			*gm.*	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*		
Mar. 26	80	12.24— 1.28	6.70	7.34	0.664	4.84					
		1.28— 2.28	6.16	6.70	0.669	4.24					
					0.667						
Apr. 14	68	12.48— 1.48	4.93	4.17	0.860	7.48	0.103			0.875	2.73
	Dog XVIII	1.48— 2.48	4.80	4.28	0.816	6.76	0.103			0.819	2.73
Apr. 17	70	12.40— 1.40	10.10	5.14	0.842	11.83	1.440	−3.36	−3.45	0.708	38.17
	Dog	1.40— 2.40	10.45	5.32	0.845	11.94	1.440	−3.01	−3.18	0.688	38.17
	XVIII	2.40— 3.40	10.56	5.38	0.845	10.69	1.440	−2.90	−3.08	0.685	38.17
					0.844					0.694	
Apr. 19	81	3.04— 4.04	6.45	7.03	0.667	4.57					
		4.04— 4.44	4.46	4.80	0.676	3.06					
					0.670						
1922											
Mar. 6	141	1.41— 2.41	8.17	8.95	0.664	6.14					
		2.41— 3.41	8.09	8.93	0.659	5.87					
		3.41— 4.41	8.01	8.75	0.666	5.78					
					0.663						
Mar. 8	142	11.26—12.26	8.33	8.98	0.674	6.12					
		12.26— 1.26	8.27	9.21	0.653	5.94					
					0.664						
Mar. 8	75	2.50— 3.50	11.54	10.36	0.810	13.86	1.80	−5.32	−4.88	0.786	47.80
	Dog XIX	3.50— 4.50	11.73	9.97	0.856	13.19	1.80	−5.13	−5.27	0.708	47.80
					0.833					0.749	
Mar. 11	144	10.21—11.21	7.75	8.53	0.661	5.97					
		11.21—12.21	7.66	8.41	0.662	5.65					
					0.662						

* Calorimeter cold.

y Metabolism. Series II.

sect.	Body temperature Start.	End.	Morning weight.	Behavior of dog.	Food.
			kg.		
50					
95					
45					
76	37.81		8.57	Quiet.	Basal metabolism.
31		37.45		"	
07					
52				Quiet.	1,000 gm. of meat at 8.33 a.m. 3rd day of same
07				"	diet. Also 100 gm. of biscuit meal at 5 p.m. for
73		38.48		"	previous 2 days.
32					
17					
12					
59					
24					
76					
35					
35					
36					
75					
11					
39	38.72			Quiet.	1,000 gm. of meat at 9 a.m. 3rd day of same diet.
12		38.65		"	Standard diet also given constantly at 5 p.m.
31					
35*					
78					
13					

Date.	Experiment No.	Time.	CO₂	O₂	R. Q.	H₂O	Urine N.	Non-protein.			Pro-tein.
								CO₂	O₂	R. Q.	
1922			*gm.*	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*		
Mar. 11	77	1.54— 2.54	12.85	10.92	0.856	22.65	1.803	−4.01	−4.32	0.675	
		2.54— 3.54	13.40	11.65	0.837	19.85	1.803	−3.46	−3.59	0.701	
					0.846			·		0.687	
Mar. 15	145	11.34–12.34	7.63	8.30	0.669	5.67					
		12.34— 1.34	7.31	8.16	0.652	5.42					
					0.660						

In Table I it will be noted that the basal metabolism of a dog maintained upon the ordinary "standard diet" (meat, 100 gm.; biscuit meal, 100 gm.; lard, 20 to 30 gm.) was 16 calories per hour. After 8 days of meat ingestion as the sole diet, administration of the "standard diet" was resumed. 18 hours after the second administration of the "standard diet" the basal metabolism was 19.7 calories and then on successive days was measured as being 18.3, 17.3, 18.2, and 17.6 calories. Even after 2½ weeks the basal metabolism was 17.08 calories, showing a persistently higher level than had obtained before the ingestion of meat.

The bodily condition is always a factor to be considered in the determination of basal metabolism. The condition of muscular strength, accompanied by the addition of an "improvement quota" of protein (4) to the cells of the body, results also in a higher metabolism in the dog (5) and in man (6). It is possible that a lesser amount of "deposit protein" and of "improvement protein" may be in part responsible for the lower basal metabolism of women, first pointed out by Gephart and Du Bois (7). Thus the recent experiments of Blunt and Bauer (8) show that under-nutrition does not play a large part in reducing the metabolism of women.

The Experiments of Rapport and Lusk.

The problem of the production of fat from protein seemed of sufficient significance to endeavor to follow the same with the

led.

	Body temperature.		Morn-ing weight:	Behavior of dog.	Food.
)irect.	Start.	End.			
			kg.		
6.47	39.04		13.0	Quiet.	Meat 1,000 gm. at 9 a.m. 6th day of same diet at
6.44		28.76		"	9 a.m., with standard diet also daily at 5 p.m.
2.91					
8.93					
5.65					
3.58					

greatest care as to the accuracy of the determination of the individual respiratory quotients after meat ingestion.

An additional procedure was added in this series. The standard diet, containing 70 gm. of starch, was given every evening at 5 p.m. in order to charge the glycogen reservoirs of the body. The meat was given early in the morning. The complete data are to be found in Table II. The accuracy of the experiments may be gauged from the following figures:

Date.		R. Q.	R. Q. of retained material
1920			
Mar. 26	Alcohol check.	0.667	
Apr. 17	Meat ingestion.	0.844	0.694
" 19	Alcohol check.	0.670	
1921			
Mar. 6	" "	0.663	
" 8			
a.m.	" "	0.664	
p.m.	Meat ingestion. ·	0.833	0.749
Mar. 11			
a.m.	Alcohol check.	0.662	
p.m.	Meat ingestion.	0.846	0.687
Mar. 15	Alcohol check.	0.660	

If protein is converted into fat two conditions must be ful-filled: (*a*) the R. Q. must be higher than that of protein itself

and (*b*) there must be a retention of protein carbon. The urinary nitrogen is usually higher in the dog when the animal is catheterized hourly (1) than it is when it accumulates in the bladder during a calorimeter period, and the true index of protein metabolism is accounted to be the level to which the urinary nitrogen rises when the urine is fractionated hourly on a day that the dog is given the same diet but is not in the calorimeter.

The method of calculation of the 5th hour after giving 1,000 gm. of meat to Dog XVIII will suffice as a guide to all the experiments.

Experiment 68.

Urinary N = 1.44 gm.

	CO_2 gm.	O_2 gm.	Calories.
Equivalent of 1.44 gm. of N..................	13.46	12.17	38.17
Found in respiration........................	10.10	8.72	
	3.36	3.45	11.32

Calories indirect.. 26.85
R. Q. of deposit = 0.708
Value of fat deposited = 11.32 calories
Calories (indirect) = 26.85
Calories (direct) = 27.52
C retained = 0.92 gm. $\begin{cases} = 1.2 \text{ gm. of fat} \\ = 2.3 \text{ gm. of glucose (8.63 calories)} \end{cases}$
Calories if C had been retained as glycogen = 29.54

The respiratory quotients and the heat directly measured confirm the conception that fat and not glycogen was the form in which the carbon was deposited.

If one makes use of this method of calculation for the average hourly heat production for the series of three experiments, one may construct Table III.

It is evident that, computed on the oxygen absorption by the method of indirect calorimetry already outlined, the calculated heat production is almost exactly the same as it is when the computation is based upon the hypothesis that the carbon retained is laid down in the form of fat (1 gm. of C retained as fat = 12.31 calories).

The agreement between direct and indirect calorimetry is not as close as one would wish, though in this regard the alcohol checks agree on the average within 1 per cent. The calories as calculated are 3.8 per cent less than the calories found. However, if the carbon retention is assumed to take the form of glycogen, then the calculated heat would be 6.3 per cent higher than the amount directly measured. The findings, therefore, favor the idea of the retention of a pabulum in the form of fat.

TABLE III.

Direct and Indirect Calorimetry in Hourly Periods after the Ingestion of 1,000 Gm. of Meat.

	Experi-ment No.	Urine N.	Protein C to body.	Calories.			
				Direct.	Indirect.	C deposited as fat.	C deposited as carbo-hydrate.
		gm.					
Dog XVIII..	68	1.44	0.84	28.44	27.55	27.83	30.30
Dog XIX....	75	1.80	1.40	32.16	30.97	30.51	34.57
	77	1.80	1.02	36.46	34.83	35.25	38.29
Total........				97.06	93.35	93.59	103.16

Here, as in the first series of experiments, the height of the metabolism is not proportional to the quantity of protein carbon retained. In Experiment 77, high humidity in the calorimeter probably produced increased respiratory activity and in consequence increased metabolism. The older literature has been elsewhere reviewed (9), but these experiments, by a new method, add another link to the chain of evidence that protein may be converted into fat. It must be remembered, however, that the conditions were exceptional, in that the animals were maintained upon a nutritive plane which would fill the glycogen reservoirs, were given meat up to the limit of their willingness to consume it, and were kept in absolute rest in a calorimeter at an environmental temperature of 25°C. Under these circumstances fragments of protein metabolism, which would ordinarily have been oxidized or converted into glucose and laid down as glycogen, found no other pathway open than conversion into fat. Under a natural diet these conditions would not exist.

SUMMARY.

1. When the glycogen reservoirs of the body are low the ingestion of meat in large quantity results in the deposition of glycogen.

2. The continued ingestion of much meat brings about the retention in the body of a pabulum consisting partly of glycogen and partly of fat. Only when meat in very great excess is given is fat alone retained.

3. When a carbohydrate-containing meal is given in the evening and 1,000 gm. of meat in the morning, then during the height of protein digestion the respiratory quotient indicates a production of fat from protein.

4. Following the prolonged ingestion of meat in large amounts, which induces the retention of "deposit protein," the basal metabolism may rise from a former level of 16 calories per hour to one of 19.7, an increase of 23 per cent, from which level it slowly falls with the gradual elimination of "deposit protein."

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THE LIPOIDS OF THE BLOOD IN TUBERCULOSIS.

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The investigation of fat metabolism in the body has involved the study of the fats and other lipoids as they occur in the intestine, the blood, and the tissues. In the blood (1) the lipoids are found normally in a fairly constant quantitative relationship to each other. The work of Brinkman and van Dam (2) has recently emphasized the physiological antagonism between lecithin and cholesterol, which was found by earlier workers (3). Bloor (3) and others have shown that whenever one of the blood lipoids is high, similarly high values are to be expected for the others; and especially as regards lecithin and cholesterol is the balance carefully preserved. An extension of this study to diseases of a metabolic nature has led Rudolf (4) to consider the lipoid picture as of considerable diagnostic value, because he found that in each disease he studied there was a definite change from the normal. Special studies have been made of the blood lipoids in diabetes (5), nephritis (6), and the anaemias (7). Nowhere has any attention been paid to the condition of the blood lipoids in tuberculosis, although certain phases of this disease would lead one to expect some abnormality of fat metabolism. Thus there is a toxemia with resulting emaciation, formation of the characteristic tubercles which contain much lipoid material, and the use in treatment of a fat-rich diet. It seemed desirable, therefore, to extend the study of the blood lipoids to tuberculosis and in the following paper are reported observations made on a series of twenty-one cases of advanced tuberculosis, mainly pulmonary.

Subjects.—All the subjects were patients in the tuberculosis wards of the San Francisco Hospital. All had chronic pulmonary

tuberculosis, advanced, giving negative Wassermann reaction and were apparently free from intercurrent infections. Blood from Cases 1 to 9 inclusive was drawn October 22, 1921, and from the remainder December 15, 1921. Cases 2 and 11 are the same man. His blood was used a second time to check the method.

TABLE I.

Lipoids in the Blood in Tuberculosis.

Cases.	Cholesterol.		Lecithin.	Fatty acids.				Lecithin/Cholesterol	Cholesterol/Fatty acid
	Saponi-fication.	Non-saponi-fication.		Total.	Fatty acid in lecithin.	Fatty acid in choles-terol esters.	Resid-ual fatty acid.		
1	116	202	203	380	134	67	179	1.00	0.53
2	82	190	216	362	144	63	155	1.13	0.52
3	126	168	203	336	134	56	146	1.20	0.50
4	104	244	240	398	160	81	157	0.99	0.61
5	138	236	227	362	152	78	132	0.96	0.65
6	106	202	221	380	146	67	167	1.09	0.53
7	136	250	245	392	162	83	147	0.98	0.63
8	198	214	226	396	150	71	175	1.05	0.54
9	112	174	206	348	136	58	154	1.18	0.50
10	86	169	227	346	150	56	140	1.34	0.46
11	91	228	240	375	160	76	139	1.05	0.60
12	90	219	214	362	142	73	147	0.97	0.60
13	94	201	229	344	152	67	125	1.13	0.58
14	108	206	207	372	138	68	166	1.00	0.55
15	76	174	202	339	134	58	147	1.17	0.51
16	82	186	205	369	136	62	171	1.10	0.50
17	90	196	240	378	160	65	153	1.22	0.51
18	90	232	204	361	148	77	136	0.87	0.64
19	97	214	217	375	144	71	160	1.01	0.57
20	96	198	214	363	142	66	155	1.08	0.54
21	99	180	192	334	128	60	146	1.06	0.53

Cases 7 and 8 were discharged from the Hospital as improved. Case 21 was very weak and emaciated, the lowest results were obtained from him.

Methods.—The methods which were used for this work for the quantitative determination of the blood lipoids are those described in the various contributions of Bloor (8, 9, 10). Determinations were made only on the plasma since the experience of workers in this field has shown that, except in alimentary lipemia, the lipoid

composition of the corpuscles is relatively constant. Cholesterol was determined on the blood extract both with and without saponification since it was found that values by the two methods were often strikingly different.

Calculations.—Values for cholesterol, fatty acid, and lecithin were obtained directly while the remaining values were calculated from these. Fatty acid in cholesterol esters was calculated to be one-third of the total value of cholesterol determined by the non-saponification method, while that in lecithin was taken as two-thirds. The sum of these two values subtracted from the value for total fatty acids gives the amount of fatty acid in combinations other than those mentioned. The ratios, $\frac{\text{lecithin}}{\text{cholesterol}}$ and $\frac{\text{cholesterol}}{\text{fatty acid}}$, were calculated only for the non-saponification values of cholesterol. Values expressed in milligrams per 100 cc. of plasma are contained in Table I.

DISCUSSION.

Cholesterol.—Cholesterol was found by the non-saponification method to be practically normal, but the saponification method gave results uniformly much lower (averaging about 50 per cent) except in Case 8, and it is possibly significant that in this case improvement had occurred and the patient was allowed to leave the hospital. The difference between the values for cholesterol by the two methods is very striking and since ordinary cholesterol is not appreciably affected by saponification the presence of some substance other than cholesterol which gives the cholesterol color reaction but which is sensitive to alkali is indicated. Since the value found without saponification is the same as similar values for normal blood it appears that in tuberculosis true cholesterol is replaced in considerable part by the unknown substance, which may, however, be closely related to cholesterol since it gives the same color reaction. The reason for the apparent substitution for cholesterol in tuberculosis blood is at present a matter of speculation, but when the lipoid nature of the tubercle bacillus and of the tubercles is considered, it seems reasonable to suspect that cholesterol may be involved in some way in the attempt of the body to protect itself from this organism.

Lecithin and Fatty Acid.—These substances were present in amounts comparable with values for the normal published by Bloor. The residual fatty acid is relatively high and since there was no visible lipemia the fatty acids could hardly be present as fat, so that some other form of combination is suggested. The ratios, $\dfrac{\text{lecithin}}{\text{cholesterol}}$ and $\dfrac{\text{cholesterol}}{\text{fatty acid}}$, are fairly constant for the entire series. They do not vary widely from those given for normal individuals.

CONCLUSIONS.

1. Cholesterol was found to be uniformly low in tuberculous blood when determined by the saponification method, but normal when determined without saponification. The presence in the blood plasma in tuberculosis of a relatively large amount of an unknown substance which is probably closely related to cholesterol is thus indicated.

2. Total fatty acid and lecithin were within the normal range of values for these substances.

3. The "residual" fatty acid of the blood was high and since there was no lipemia the presence of other forms of fatty acid combination than those ordinarily present is suggested.

Thanks are due Dr. W. R. Bloor for his kind supervision and ready assistance, and to Drs. Wm. H. Hassler, Ester Rosencrantz, and W. H. Yokum, who made it possible to secure the blood and data for this work.

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FAT EXCRETION.

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The feces of animals normally contain some fatty material which consists mainly of fatty acids and their salts (soaps), a smaller amount of cholesterol and its derivatives, and a little fat. The fatty acids and fats are generally assumed to be unabsorbed residues of the fat of the food and a food fat is said to be well or poorly absorbed according as the amount of fatty substance recoverable from the feces is relatively small or large. In some cases, and especially when the amount is large, there is little doubt that the assumption is approximately correct and that the fat and fatty acids found represent largely unabsorbed food fat, but there is considerable evidence to indicate that in many or perhaps most instances the feces fat has no direct relationship to the fat of the food, but represents rather some form of excretion from the intestinal tract. Friedrich Müller (1) in studies of the feces fat of the two professional fasters Cetti and Breithaupt found in the case of Cetti that the fatty material of the fasting feces was about 36 per cent of the dry material and that 39 per cent of it was fat, 45 per cent fatty acids, and 16 per cent cholesterol. In the case or Breithaupt the fatty material composed 28 per cent of the dry matter and of this, neutral fat and cholesterol formed 47 per cent, fatty acids (and soaps) 53 per cent. In two periods with food on the same subject the lipoid material formed, in the first period, 24 per cent of the dry material, and of this 36.5 per cent consisted of neutral fat and cholesterol and 62.6 per cent of fatty acid. In the second food period the lipoid material constituted 28 per cent of the dry substance, and of this 29 per cent was neutral fat and 71 per cent fatty acid and

soap.   The percentage of total lipoid material in the feces during the food period thus differed but little from that of the fasting period, although the proportion of fatty acid and soap was relatively considerably higher during the food period.   Also, since the amount of feces was greater, the absolute amount of lipoid material excreted during the feeding period was greater than during the fasting period.   In the same article Müller refers to some earlier experiments (2) with dogs in which he found a lipoid content in the feces of 20 to 47 per cent of the dry substance, consisting mainly of fatty acid.   Thus in one dog (2) weighing 23 kilos, in a 7 day fast the excretion was about 2.68 gm. of dry material per day, of which the fatty material amounted to about 34 per cent, and consisted of 67 per cent of fatty acid (and soap) and the remainder of neutral fat and cholesterol.   He believed that this material originated as an excretion of the intestine and pancreas.   In another dog weighing 18 kilos, on a diet of lean meat the feces amounted to 6.1 gm. of dry matter per day and contained 25 per cent of fatty material, of which 62 per cent was fatty acid and 38 per cent cholesterol, fat, etc.   The addition of small amounts of fat to the diet affected the lipoid content of the feces only slightly, but larger amounts increased the lipoid output. The latter finding is not, however, incompatible with his assumption of a fat excretion, since where large amounts of fat are ingested a larger excretion might be expected.

Hermann (3) isolated loops of intestine and found that they filled up in the course of 3 or 4 weeks with material very similar to feces, and an examination of this material by Ehrenthal (4) demonstrated the presence of fat, soaps, and cholesterol.   F. Voit (5) repeated the work and confirmed their results, finding that the contents of the intestinal ring had the same composition as hunger feces and almost the same as meat feces.   In addition to ash and nitrogen there was always fatty material to the extent of 30 to 36 per cent, of which up to one-third (generally less than one-tenth) was neutral fat, one-half to four-fifths free fatty acid, and one-tenth to one-third soaps.

By the use of fistulas further information regarding intestinal secretion was obtained.   Gumilewski (6) obtained from a low Thiry-Vella fistula in dogs a continuous secretion which was small in amount in fasting, 1 cc. per hour from an 11 cm. length of

intestine, but increased to 7 to 10 cc. during digestion. Röhmann (7) found little or no secretion from a high loop while considerable secretion was obtained from a low loop. Some time ago one of us (B) examined the secretion from a permanent Thiry fistula (consisting of about 14 inches of jejunum) in a healthy dog.[1] The secretion was collected on fat-free pads of gauze which were then boiled out with alcohol to extract the lipoid material. The alcoholic extracts were evaporated to small volume, diluted with water, acidified, the fatty matter was extracted with ether, the solvent evaporated, and the residue dried and weighed. In a 5 day period a total of 0.72 gm. of lipoid material consisting almost entirely of fatty acids was recovered. The animal was well fed during the period. It was not possible to repeat this important experiment at the time and the dog was disposed of. Numerous attempts have been made since then to obtain suitably operated animals but none survived the operation and resulting complications long enough to be used for an experiment.

The results noted above indicate that much fatty material is to be found in the intestine and feces which are entirely independent of the food. The question as to whether it is to be regarded as a secretion is complicated by the fact that in the feces and in intestinal loop contents there is much cellular material (bacteria, etc.), which contains fatty compounds and contributes to the "fat" content. The importance of this source of fat cannot be estimated. As regards the contents of intestinal rings, Voit (5) does not regard it as important since the nitrogen content is too low and the ash too high to be of cellular origin. The fact that the free flowing secretion from the intestinal fistulas contains little cellular material but considerable soap points to a true secretion.

Aside from the undetermined influence of lipoid from cellular material it is reasonable to assume that if the fatty material of the feces represents a true excretion of the intestine its nature would be independent of the food and of the food fat. If it represents unabsorbed food residues its nature would depend on that of the food fat. Certain modifications of this general assumption should probably be made in view of the probability

[1] Obtained through the kindness of Dr. Barney Brooks of the Washington University School of Medicine, St. Louis.

that on the one hand some of the food fat after absorption may be excreted into the intestine and on the other that the absorption of food fat from the intestine may be selective, less desirable, and possibly similar portions of all types of fat being rejected and appearing in the feces.

With these ideas in mind feeding experiments were carried out with two fats of widely different composition, and the feces fat was examined. Cats were used for the experiments. They were kept in cages throughout the period but allowed the free run of the room during a portion of the day for exercise. Their basal diet was a practically fat-free mixture of starch and extracted casein with meat extract for flavoring and bone ash to provide bulk. Experiments were conducted with (a) a diet of lean meat, (b) the basal diet alone, (c) the basal diet plus olive oil, (d) the basal diet plus coconut oil; in every case making the amount of the daily food such as to supply 100 calories per kilo of body weight. Generally the food was entirely eaten up, but occasionally when olive oil was fed and more frequently with coconut oil it was necessary to feed forcibly a portion of the oil in order to make sure that the cats received the required amount. Although apparently remaining in good health the animals lost weight steadily throughout the experiment.

The meat used contained from 2 to 7 per cent of fat with an iodine number of about 46 and a melting point of about 43°C. The casein was mainly prepared fresh from skim milk, but some commercial casein was used. In preparing it for use all samples were extracted with alcohol and ether and as used the casein-starch basal diet contained only a few milligrams of fatty substance in 100 gm. The coconut oil was fed in the form of the commercial butter substitute "Nucoa." This substance consists almost entirely of coconut oil but the flavor is somewhat disguised and is less objectionable than that of the commercial oil. The fatty acids of the material used melted at about 25°C. and had an iodine number of 8.8. The olive oil had an iodine number of 88.2.

Each experiment lasted a week, the periods being marked off by charcoal. The feces were collected as passed, kept in a stoppered bottle under 95 per cent alcohol until the end of the period when the whole was transferred to a large Erlenmeyer

TABLE I.

Feces "Fat" on Various Diets.

Subject No.	Fat eaten.	"Fat" in feces.	Percentage of "fat" in feces to fat in diet.	Iodine No. of feces "fat."	M.P. of feces fat.
	gm.	*gm.*	*per cent*		*°C.*
Fat-free diet.					
1		1.70		36.3	38
		3.29		32.6	42
2		0.86		37.1	26
3		1.47		27.4	39
4		1.47		30.0	30
Meat diet.					
1	50	6.00	12.0	39.0	44
	28	0.87	3.1	43.8	34
2	34	2.37	6.9	34.8	33
3	28	1.10	3.9	42.3	36
4	28	3.84	13.7	30.5	35
Coconut oil.					
1	44	2.17	5.1	25.0	32
2	45	2.46	5.5	24.4	34
3	24.3	1.82	7.6	31.0	30
4	52	3.55	6.9	21.6	27
Olive oil.					
1	30	1.50	5.0	38.7	30
	50	2.00	4.0	53.2	26
2	45	2.51	5.6	41.0	32
	50	1.81	3.6	44.8	30
3	50	2.76	5.5	49.8	33
	50	1.33	2.7	52.2	25
4	50	3.0	6.0	44.3	37
	50	3.0	6.0	32.7	40
Averages.					
Fat-free diet.....		1.76		32.7	35
Meat diet		2.83	7.9	38.1	36
Coconut oil.......		2.50	6.3	24.8	31
Olive oil.........		2.24	4.8	44.6	31

flask. Alcohol was added to cover the material, then 10 gm. of stick potassium hydroxide, after which the flask was connected with a reflux condenser and boiled for 5 hours. At the end of the time the mixture was diluted with water, acidified with hydrochloric acid, and completely extracted with ether. The extracts were united, washed with water, the ether was distilled off, and the residue dried in a vacuum desiccator for 24 hours. The residue was then extracted with petroleum ether, the extract filtered, the ether distilled off, and the fatty residue dried as before and weighed. In this series of experiments no account was taken of the cholesterol-like substances (unsaponifiable matter). In determining their fat content the food materials were treated in the same way as the feces. Iodine number determinations were made by the Wijs method. Five experiments were carried out with the fat-free and meat diets, four with coconut oil, and eight with olive oil. The results of the experiments are given in Table I.

DISCUSSION.

Total Fat.—The total "fat" in the feces varies a great deal on all the diets so that the average has not much meaning; but inspection of the data on the experiments will show that, as would be expected, there is less fat in the feces on the fat-free diet than on the others. Of the other three the meat diet yields the largest amount, due probably to enclosure of fat by the tissue, and the olive oil the least. Probably for the same reason (enclosure) the percentage feces fat of food fat is highest on the meat diet.

Iodine Number.—The iodine number is highest on the olive oil diet and lowest on the coconut oil, but in neither case does it approach the values of the fat fed, having rather a value not so greatly different from that of the fat-free diet. It is plain that the fat of the food has some influence on the feces fat but the influence is not great, especially when the amounts of fat fed are moderate, as in these experiments.

Melting Point.—The melting point of the feces fat is relatively constant, but is consistently lower on the fat diets than on either the fat-free or the meat diet, showing again the influence of the fat of the diet on the feces fat. The melting point of the feces fat is almost always below body temperature.

These results show, in agreement with the work of earlier investigators, that "fat" is to be found in the feces whether it is present in the food or not, and that fat in the food increases the feces fat, but only to a comparatively small extent. They show further that the nature of the "fat" of the feces is to a great extent independent of the food fat, being much the same no matter which of two widely different fats were fed or whether none was fed at all. Taken altogether they indicate a continuous output of "fat" in the feces of a constant composition independent of the diet.

The constancy of the feces fat independent of the diet favors the idea of a fat excretion, but might, of course, be explained as due to a constant output of cellular waste from the intestine and from bodies of bacteria. The answer of Voit to this possibility (see above) is not convincing, since the presence of much cellular material in the feces cannot be denied. The fact that fatty material can be collected from isolated portions of the intestine under conditions which to a large extent exclude a cellular origin (Thiry fistula) is a better answer to the objection, but unfortunately there is not much evidence of this nature available. It is not unlikely that feces fat may have more than one origin just as the fatty material from the skin comes partly from desquamated epithelium, and partly from the sebaceous glands.

SUMMARY.

When moderate amounts of fat are fed the fat of the feces is largely independent of the diet, and in composition approaches that from a fat-free diet.

The comparative constancy of composition of the feces fat favors the idea of a fat excretion from the intestine but while an excretion is probable it cannot be regarded as proven in view of the undetermined influence of lipoid from free cellular material.

The feces fat cannot ordinarily be regarded as unabsorbed food fat and, therefore, feeding experiments as a test of the extent of utilization of food fat are of doubtful value unless account be taken of the amount and kind of fat which appears in the feces independently of the food.

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ON THE COLORIMETRIC DETERMINATION OF HEMO-GLOBIN WITH ESPECIAL REFERENCE TO THE PRODUCTION OF STABLE STANDARDS.

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CONTENTS.

INTRODUCTION.

Sahli demonstrated as the underlying principle of his well known hemometer, that the color intensity of an acid hematin solution varies directly as the amount present, a relation which does not, however, hold for all colored compounds. This allows the preparation of solutions of any desired strength from more concentrated ones according to the simple rules employed in volumetric work. But owing to a certain lack of permanence of all dilute acid hematin solutions, new ones must be prepared at intervals depending to some extent upon the method of preparation and preservation. These are best standardized by comparison with blood whose hemoglobin has been determined by the oxygen capacity method of Van Slyke, as recently modified by Van Slyke

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and Stadie (1). The latter method, although very accurate, requires considerable experience in order to obtain consistent results. Attempts have therefore been made to devise a standard which limits as far as possible both the number and frequency of such checks.

Cohen and Smith (2), and later Robscheit (3) employed concentrated stock solutions of acid hematin from which dilutions were prepared at frequent intervals.

The method as described by these authors has several objectionable features. On standing, the stock solution deposits a sediment, and dilutions are always very turbid, more so when prepared from old solutions; also changes in color can be demonstrated often within 2 months, sufficient to prevent accurate color matching. Fading occurs to a variable degree, although the turbidity and color change after a time make it difficult to estimate. Accordingly, various modifications were tried in attempts to produce a more satisfactory stock solution, of which the following is recommended.

Production of Standards.

1. *Stock Solutions of Acid Hematin.*—Defibrinated or oxalated blood is centrifuged, the serum removed, sufficient water added for laking, and hydrochloric acid added to a concentration of 0.1 N. This is allowed to stand for 24 hours at room temperature for full color development. The solution formed corresponds very well to that prepared by the method of Cohen and Smith, or Robscheit, but the absence of the greater part of the serum proteins favors concentration as described later. The solution so prepared is distinctly turbid, but by repeated filtration, preferably through hardened papers, a perfectly clear solution is obtained, having a concentration of from 7 to 12 per cent depending upon that of the unfiltered preparation, and showing only a very slight degree of sedimentation even after standing for a week.

Still more concentrated solutions, however, are desirable both from the standpoint of stability and convenience. Since heating is injurious, concentration is best accomplished by evaporation with an electric fan, the solution being poured into a flat dish. The process requires several hours and should be carried out in as dust-free an atmosphere as possible. When the solution has reached a syrupy consistency it is again filtered, this time by suction, in order to remove any dust particles taken up during evaporation. Evaporation is continued to the thickest solution that can be measured with a pipette, usually corresponding to from 15 to 20 per cent according to Haldane's scale. A known dilution is then standardized

against a known acid hematin solution and the concentration of the stock solution calculated. It is then diluted to a convenient point, preferably not below 15 per cent, and for later convenience accurately measured into 2 cc. brown glass ampules. Care must, of course, be taken, owing to the viscosity of the solution, to allow the pipette to drain very slowly.

Preservation.—The more highly concentrated solutions require no preservative, some specimens having stood in the laboratory for several months without decomposition. More dilute solutions may show a growth of mold after long standing, but this can be prevented by the addition of from 10 to 20 per cent of glycerol.

TABLE I.

Percentages of Color Retained by Stock Solutions after Sealing in Ampules.

Since sealing.	Color retained.		
	Solution 1.	Solution 2.	Solution 3.
mos.	*per cent*	*per cent*	*per cent*
1	98	98	97
2	96	97	97
3	96	97	96
4	95	96	96
5	95	95	95
6	95	95	95
7	94	95	95
8	94	94	94
9	94	94	94
10	93	92	93

Dilutions made from these ampules, of a strength usually employed for colorimetric work, are perfectly clear, and show only the slightest tendency to sedimentation. This may be prevented almost indefinitely by incorporating from 10 to 20 per cent of glycerol in the diluting fluid. The glycerol furthermore serves as an effectual preservative.

Stability.—In order to test the stability of these ampules under varying conditions, some were placed in a refrigerator, and others kept in the dark at room temperature. These were broken at intervals of a month, and tested against an acid hematin solution of known strength. The results shown in Table I are taken from those kept at room temperature, and are expressed in percentages of the strength at the time of sealing.

There was no appreciable difference in the case of those kept in the refrigerator. In both instances, fading, while more rapid during the first few months, continued slowly. At no time was any qualitative alteration of color noted.

2. Acid Hematin Protein Powder.—Since all solutions of acid hematin are obviously subject to change, and in consideration of the almost invariably greater stability of pigments in dry form, it seemed logical to assume that if the preparation could be reduced to a dry powder, from which standards could be prepared by weight, an important step toward the stability of stock preparations would be made. Attempts to prepare such a powder by continuing the evaporation of the above solutions, resulted in preparations which although giving perfectly clear solutions with almost no tendency to sedimentation, were at times soluble only with the greatest difficulty, and at others, showed sufficient deviation from the true color to prevent exact matching. The disturbing element seems to be the presence of too much inert material. The following method, which resembles very closely that employed by Williamson (4) for preparing purified solutions of hemoglobin was found satisfactory for its removal.

Preparation.—Several hundred cc. of defibrinated or oxalated blood are centrifuged, the serum is removed, and the cells are washed four times with normal saline solution. The washed cells are mixed with an equal volume of distilled water, sufficient ether is added to produce complete laking, the mixture thoroughly shaken and allowed to stand 10 minutes. It is filtered or centrifuged to remove the stroma of the red cells, first diluting slightly with distilled water if too viscid. The filtrate is a clear dark red liquid still containing variable amounts of protein. This is removed by the addition of an equal volume of aluminum cream prepared according to the method of Tracy and Welker (5), followed by filtration by suction or preferably centrifugalization. Alcohol is then added in small portions with constant shaking, until it constitutes about 20 per cent of the volume. An appreciable precipitate here would indicate that all the serum protein had not been removed, although a slight turbidity almost always results from the action of the strong alcohol before being sufficiently diluted. In either event the solution should be again filtered through a hardened paper, preferably without suction.

Air or oxygen is then blown through the solution until the hemoglobin is completely saturated, after which ½ volume of 0.25 N hydrochloric acid is added in small amounts with constant shaking. Instead of the turbid solution obtained by the addition of acid directly to whole blood, a very dark sometimes syrupy, but perfectly clear product results. At least 24

hours should be allowed for full color development. Under no circumstances should it be warmed, since it may change to a gelatinous mass soluble only with great difficulty after drying.

Evaporation should be carried out in the same manner as above described, and when the consistency of a thick syrup is reached, the solution is again filtered as before through a hardened paper. Evaporation to dryness is then completed as rapidly as possible, the mixture being frequently stirred. If facilities are available, evaporation may be most rapidly completed over phosphorus pentoxide under greatly reduced pressure, a procedure which furthermore precludes any possibility of contamination by dust particles. When thoroughly dry, the entire bulk of the preparation should be very finely powdered and thoroughly mixed, in order to facilitate solution as well as to insure homogeneity. The yield from 500 cc. of normal blood is about 40 gm.

Characteristics.—The resulting product is a dark brown, hard, brittle, vitreous mass, rather easily soluble in distilled water, but much less so in 0.1 N hydrochloric acid. In either instance it forms a perfectly clear solution, the color of which exactly matches that of a freshly prepared solution of acid hematin. It is stable in air, and does not absorb more than 0.1 per cent of moisture when exposed. If perfectly dry, heating to 100°C. for several hours does not affect it, but high temperatures (160°C.) render it insoluble. The age of the oldest batch is now over 3 months, and so far no alteration of any of its properties has been noted.

No attempts have been made at analysis, since the product is obviously a mixture of so called "acid hematin" with the protein of the broken hemoglobin molecule. It has been said that on the conversion of hemoglobin into acid hematin, the latter constitutes approximately 4 per cent, while the protein fraction constitutes 96 per cent of the resulting products. All attempts to remove the protein fraction by precipitation have failed owing to the simultaneous precipitation of the pigment. It is difficult to determine the exact state in which the latter exists, but if we accept the usual statements as to its insolubility in water or hydrochloric acid, we must suppose it to be in colloidal form, the maintenance of which is aided by the relatively large amount of protein. This assumption receives further support from its failure to dialyze through a parchment membrane.

Solutions made with 0.1 N hydrochloric acid differ further from the substance commonly termed "acid hematin" in that even

in concentrated solutions, no distinct bands are visible in its spectrum, but only a diffuse absorption approaching the yellow from both sides.

The most striking characteristic of the product is, however, a remarkable uniformity of color of specimens made at different times from different kinds of blood. One might be led to expect this however, since it is made under identical conditions from comparatively pure solutions of hemoglobin. Although the composition of hemoglobin undoubtedly varies in the types investigated, this does not seem to be of a character or degree sufficient to affect the color of the solution for colorimetric work.

TABLE II.

Comparative Color of Different Preparations of Powder Containing 1 Mg. per Cc.

Blood.	Source.	Standard setting.	Unknown reading.
		mm.	*mm.*
1	Human.	20	20.0
2	Sheep.	20	19.9
3	"	20	19.9
4	"	20	20.0
5	"	20	20.2
6	Dog.	20	20.1

In order to demonstrate this uniformity of color, solutions containing 1 mg. of dry powder per cc. were prepared according to the method to be described later, from six different batches of powder, which had been made at different times, from sheep, dog, and human blood, as indicated in Table II. No. 1 was arbitrarily chosen as a standard with which the others were compared. The results are given in Table II.

It would seem that these results obviate the necessity of checking by Van Slyke's oxygen capacity method.

In order to demonstrate further this constancy of color, two solutions, each containing 1 mg. of the dry powder per cc., were made up as described under "Preparation of standard solutions," from each of five different batches of powder. Each solution was read against 0.5 per cent acid hematin solutions prepared as described under "Procedure for hemoglobin determination," from each of

fifteen different bloods whose hemoglobin had been previously determined by Van Slyke's oxygen capacity method. From these data, calculations were made of the amount of dry powder per cc. of solvent required to give a color corresponding to a 1 per cent solution of acid hematin prepared from blood containing 10 gm. of hemoglobin per 100 cc. The average value was 1.48 mg. per cc., the highest being 1.51, and the lowest 1.46.

It is thus seen that 1.48 mg. of the powder are required to give the same color as 1 mg. of hemoglobin after conversion into acid hematin. One might expect the amounts to be more nearly equal in view of the relative purity of the hemoglobin solution from which the powder is prepared. The cause of this difference is not quite apparent and will require further investigation.

Standardization.—The powder is prepared for standardization by dissolving a carefully weighed portion, usually between 30 and 50 mg., in sufficient distilled water to make a solution containing 2 mg. per cc., and then adding an equal volume of accurate 0.2 N hydrochloric acid. Heating is to be avoided. Owing to slight color changes which may follow the addition of the acid, it is allowed to stand for an hour before reading. This is done against a solution of known strength made from blood previously laked with distilled water as later described. From this comparison, the number of mg. of powder per cc. required to give a solution of any desired strength can be readily calculated.

Preparation of Standard Solutions.—Standard solutions to be used regularly in the laboratory should contain from 10 to 20 per cent of glycerol in order to prevent any slight sedimentation or the possible growth of molds. This is best added directly to the water, and a measured quantity of the mixture used. In order to prepare a standard, the powder is weighed to 0.1 mg. and the required amount of solvent calculated. It is completely dissolved in half this volume of the above mentioned glycerol solution, after which an equal amount of accurate 0.2 N hydrochloric acid is added. After standing an hour it is ready for use. If kept at room temperature in tightly stoppered brown glass bottles, it remains unchanged for from 4 to 6 weeks, but after this, slight fading will have occurred, although deviation from the true color has rarely been noted even after much longer periods. The simplicity of such a method is obvious, and since sufficient material

can be obtained from 500 cc. of blood to supply a large laboratory or hospital for months, frequent preparation and standardization are avoided.

3. Acid Hematin Films.—Because of the generally greater stability of dry preparations, attempts were made to prepare transparent films of acid hematin which could be employed in the same manner as Newcomer's glass plate. The following method will be found convenient.

A concentrated aqueous solution is prepared from the powdered acid hematin, and sufficient hydrochloric acid is added to bring the acidity to about 0.1 N. This is added to a moderately thick solution of gelatin previously filtered and cooled to about 60°C. After thorough mixing, with care to avoid the formation of air bubbles, it is poured on carefully cleaned 35 × 55 mm. cover-glasses. After draining off the excess, these are carefully leveled and placed in a dust-free place to dry.

A few trials may be necessary to obtain the optimum color, which may be varied to suit the wishes of the individual without sacrifice of accuracy. Films so made are almost perfectly uniform except at the edges. These can be trimmed off with a diamond, and squares of any desired size cut from the remainder. They are mounted in balsam on thin white microscope slides and standardized against a known acid hematin solution. A factor can easily be computed for each film, expressing readings either in percentage or grams of hemoglobin.

Compared with the colored glass plate of Newcomer, they possess the decided advantage of a much darker color, although matching perfectly that of acid hematin solutions and so allowing greater ease and accuracy of readings. Their chief disadvantage lies as might be expected, in a certain lack of permanence, yet kept in the dark they have retained their full color over a period of several months.

Turbidity.

The effect of turbidity of both standard and unknown, invariably present when prepared by the addition of whole blood directly to 0.1 N hydrochloric acid, does not seem to have been sufficiently appreciated.

It has been found impossible to duplicate the reported close agreements between colorimetric and gasometric methods on bloods of widely divergent hemoglobin content, using acid hematin standards prepared in the usual manner. Cohen and Smith (2) state that they also have found it difficult to obtain close agreement on some bloods. The factor of turbidity, which is *not* proportional to the amount of hemoglobin, is offered as at least one possible explanation of this discrepancy.

TABLE III.

Comparison of Hemoglobin Values by Gasometric and Colorimetric Methods Showing Effect of Failure to Lake Blood.

Blood.	Concentration.	Hemoglobin by colorimeter.				Hemoglobin by oxygen absorption.
		Laked.	Not laked.	Difference.		
	per cent	*gm. per 100 cc.*	*gm. per 100 cc.*	*mm.*	*per cent*	*gm. per 100 cc.*
1	0.5	10.0	11.0	1.0	10.0	10.1
2	0.5	14.1	15.2	1.1	7.8	14.3
3	0.5	12.4	13.5	1.1	8.9	12.7
4	0.5	15.2	16.4	1.2	7.9	15.0
5	0.5	9.7	10.6	0.9	9.3	9.7
6	0.5	13.4	14.3	0.9	6.7	13.5
7	0.5	11.0	12.0	1.0	9.1	11.0
8	0.5	8.4	9.4	1.0	11.9	8.5
9	1.0	10.6	10.7	1.1	10.4	10.4
10	1.0	15.8	16.8	1.0	6.3	15.5
11	1.0	13.2	14.1	0.9	6.8	13.0
12	1.0	12.9	13.8	0.9	7.0	12.8

Anyone can easily demonstrate that the addition of whole blood directly to 0.1 N hydrochloric acid gives values from 6 to 12 per cent higher than those obtained from the same blood first laked by adding it to distilled water. It is obvious that the higher readings can only be due to turbidity, since all other factors are equal.

In order to demonstrate this, two 0.05 cc. samples were taken in the same pipette from the same specimen of blood. One was delivered directly into 10 cc. of 0.1 N hydrochloric acid, and the other into 5 cc. of distilled water contained in accurate 10 cc. graduates. To the latter after complete laking, which requires

about ½ minute, 0.2 N hydrochloric acid was added to the 10 cc. mark. After standing 24 hours these were read against an acid hematin standard prepared from the powder, corresponding to a 1 per cent solution made from blood containing 10 gm. of hemoglobin per 100 cc. When readings are made with this standard against 20 mm. of the unknown, the depth in mm. is equal to the number of grams of hemoglobin per 100 cc. of the unknown blood. The results in Table III are so expressed.

It is apparent from Table III that the difference in readings on laked and unlaked bloods tends to be constant, rather than to bear a relation to the amount of hemoglobin. Using the unknown as a 0.5 per cent solution prepared by adding directly to 0.1 N hydrochloric acid, the average increase of color is seen to equal 1 mm. of the standard, or 1 gm. of hemoglobin per 100 cc. Employing the unknown as a 1 per cent solution, the difference is the same. But if the standard be of equal turbidity, which seems to be the case when similarly prepared, the error due to this factor approaches zero as the hemoglobin values approach one another.

It has been found that if the blood is first laked by adding it to an amount of distilled water equal to half the required final volume, then making up to volume with 0.2 N hydrochloric acid, that turbidity is so greatly reduced as to become scarcely discernible. By this means the variations between the colorimetric and gasometric methods may be brought within the percentage of error of colorimetric readings.

Effect of Variation of Temperature and Acid Concentration.

The effect of variations of temperature and acid concentration have been discussed by Stäubli (6), Newcomer (7), and Meulengracht (8), who offered certain alterations of technique tending toward increased accuracy. All present curves of color development, but that of Newcomer determined by spectrophotometric methods, presumably using 0.1 N hydrochloric acid at room temperature, is probably the most accurate. Berman (9) recommends boiling the acid hematin solution for 1 minute to develop the maximum color.

The employment of heat, while perhaps applicable to the Sahli hemometer for which it was recommended, is scarcely

practicable for accurate colorimetric work unless precautions are taken to standardize its application. Boiling invariably produces increased turbidity with consequent high readings. Heating to 60°C. seems to prevent this, but even so, except with great precautions results may be inaccurate. Variations due to slight differences of acid concentration which might result from using approximate acids, seems to be more apparent if solutions are heated to develop the color than if allowed to stand at room temperature.

Heating over a free flame even with a thermometer inserted may give inaccurate results, most probably due to the variable time required to reach the desired temperature. The only way in which such factors can be accurately controlled seems to be by employing an accurately standardized acid, placing a definite amount of the solution in a test-tube of standard size, and immersing for a constant length of time in a water bath at a constant temperature. For routine use this is unnecessarily troublesome, especially since accurate results may be obtained by the application of Newcomer's equation: $xy = -c$, where $x =$ the time in minutes since the addition of the acid; $y = 100-$ the percentage of color developed; and $-c = 40$.

DISCUSSION.

The employment of a stock acid hematin solution, prepared as suggested and kept in sealed ampules, may be of advantage from the standpoint of circumstances, since no analytical balance is required in the preparation of dilutions. Such stock solutions even when most carefully prepared and preserved, are not perfectly stable, and fading does occur, although since no alteration from the true color has been noted even in preparations nearly a year old, and since fading is considerably less after the first few months, an occasional check by the oxygen capacity method may be all that is required. The employment of brown glass for both stock solutions and standards seems to increase their permanence.

The powdered preparation on the other hand, has shown no change even after several months, and at present there seems to be no reason for doubting its permanency. It is stable at least under all ordinary conditions, and if dry is not affected by moderate heat.

It is remarkably constant in composition and for ordinary use, if properly prepared, need not necessarily be checked by Van Slyke's gasometric method.

Although an accurate balance is required for the preparation of standards, these need not be made except at infrequent intervals provided they are properly preserved. Known amounts of powder may be readily sealed in glass containers, and these broken when desired. This would allow one to employ the method clinically where even occasional access to a balance is possible.

Solutions prepared as indicated are of unvarying accuracy and can be duplicated at will. Furthermore, it is possible to prepare them of any desired strength by simple calculation. They may be used in any colorimeter, and are especially applicable to the wedge type owing to the absence of sedimentation. They are more stable than when prepared in the usual manner, and their permanent transparency is an undeniable advantage for all colorimetric work.

The additional step of preliminary laking of the blood sample before adding it to the acid is advisable both in standardization and routine determinations, since the time required is negligible and closer agreement of the colorimetric and gasometric methods on bloods of widely different hemoglobin content is assured.

Procedure for Hemoglobin Determinations.—A pipette containing 50 c.mm., which can readily be filled from a finger prick has been found most suitable. This is filled, the blood thoroughly mixed with 5 cc. of distilled water in an accurate 10 cc. graduate, and the pipette rinsed. After standing ½ minute to insure complete laking, 0.2 N hydrochloric acid is added to the 10 cc. mark, making a 0.5 per cent solution of acid hematin. After standing 10 to 15 minutes it may be read. To read, the unknown is placed on the left side of the Duboscq colorimeter and set at 20 mm., the readings being made with the standard.

Ordinarily a liquid standard is preferred to the gelatin film, since it permits the use of any desired depth of color. A solution corresponding to 10 gm. of hemoglobin per 100 cc. has been found most desirable, for when the unknown is made up as a 0.5 per cent solution as above described, and set at 20 mm., readings being made with the standard, the depth of the latter in mm. is equal to the number of grams of hemoglobin per 100 cc. of the unknown

blood. Corrections may then be made from Newcomer's equation, the time since the addition of the acid being known. •

This method has been used in a large number of determinations, and can be recommended as practicable, convenient, and accurate.

SUMMARY.

1. Methods are offered for preparing: (a) A concentrated stock solution of acid hematin to be kept in ampules; (b) a stable dry acid hematin protein powder of uniform color, from which standards may be prepared by weight; (c) transparent acid hematin gelatin films which may be substituted for Newcomer's glass plate.

2. The factor of turbidity invariably present in acid hematin solutions as usually prepared from whole blood is discussed, and suggestions are offered for its elimination.

3. The employment of heat for the more rapid development of maximum color has been found objectionable.

4. A method suitable for routine hemoglobin determinations is proposed.

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THE FATE OF CERTAIN SULFUR COMPOUNDS WHEN FED TO THE DOG.*

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It is a generally accepted fact that the synthesis of the sulfur-containing bile acids proceeds according to the following equations:

$$H_2CS - - - - SCH_2 \qquad CH_2SH \qquad CH_2SO_2OH$$
$$HCNH_2 \qquad CHNH_2 \rightarrow CHNH_2 \rightarrow CHNH_2 \quad \rightarrow$$
$$COOH \qquad COOH \qquad COOH \qquad COOH$$
$$\text{(Cystine)} \qquad \text{(Cysteine)} \quad \text{(Cysteic acid)}$$

$$CH_2SO_2OH$$
$$\qquad \qquad \rightarrow \text{Taurocholic, etc. (bile acids)}$$
$$CH_2NH_2$$
$$\text{(Taurine)}$$
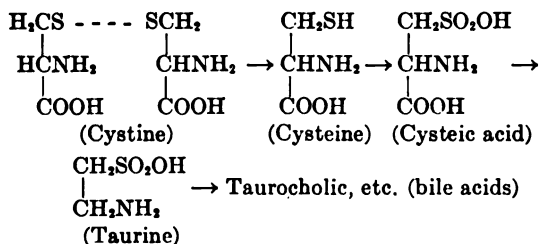
The cystine used by the body for the synthesis of bile acids is derived from the common protein catabolism and includes both endogenous and exogenous factors (1). Taurine appears to be available for combination with cholic acid at all times and hence is not the limiting factor which determines the amount of bile acids which the body can synthesize. Our experimental work with the above as well as with certain closely related compounds has been restricted to the determination of the ability of the body to oxidize the sulfur to sulfates and to utilize the nitrogen. We have also been interested in the relative rates of excretion of the nitrogen and of the sulfur of cystine and in the possible conjugation of taurine with urea to form taurocarbamic acid. The substances studied afford an excellent opportunity for the correlation of chemical structure with fate in the body. The demands of the

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body for sulfur are probably entirely supplied by cystine and not by the ingestion of other substances which we have administered to the dog. However, certain of the compounds, particularly taurine (2), occur as normal constituents of food products and are ingested in small quantities.

The experiments of Salkowski (3), Smith (4), and Heffter (5) with respect to the ability of the dog to oxidize the sulfur which is contained in sulfonic acids to sulfuric acid are at variance, and similarly, the statement that thiosulfuric acid appears in the urine when cystine is fed (6) is not confirmed by the experiments of all who have worked on this subject (7). Experiments which were carried out in this laboratory by Schmidt (8) and his co-workers show clearly that when taurine is ingested by man there is no marked increase in urinary sulfates and that contrary to the reports of Salkowski taurine is not excreted as taurocarbamic acid but as free taurine (9).

<div align="center">EXPERIMENTAL.</div>

The experimental work was carried out on dogs which were kept on constant diets containing a low but not a minimal amount of protein. The dosage of the particular substance given was added to a part of the food which was fed first. Certain of the animals, particularly Dog 1, consumed the food rapidly, the others more slowly. Urine collections were made in a metabolism cage in the usual manner and the dogs were catheterized at the end of the 24 hour period. Estimations of the sulfur fractions were carried out with the aid of the methods of Folin (10) and of Denis (11) and the amino nitrogen estimations were performed with the aid of Van Slyke's method (12). Cysteic acid was prepared from cystine according to the method described by Friedmann (13). Taurine was obtained from the abalone (*Haliotis*) by the method described by Schmidt and Watson (2). For the preparation of isethionic acid, taurine was treated with an excess of nitrous acid as suggested by W. Gibbs (14), and after acidifying and evaporating the solution, the residue was extracted with absolute alcohol from which, on cooling, the product crystallizes.

The results are given in Tables I to VI and a résumé, showing the significant figures of these experiments, is contained in Table VII. In the interpretation of the data, due consideration must be given

TABLE I.
Experiment 1. Dog 1.

Day of experiment.	Total sulfur.	Inorganic sulfur.	Ethereal sulfur.	Neutral sulfur.	Total nitrogen.	α-amino nitrogen.	Remarks.
	gm.	gm.	gm.	gm.	gm.	mg.	
1	0.291	0.159	0.030	0.103	4.23	170	Weight 13.9 kilos.
2	0.254	0.129	0.012	0.123	3.70	118	
3	0.206	0.121	0.010	0.075	3.36	90	
4	1.277	0.145	0.015	1.117	4.20	493	5.85 gm. taurine = { 1.5 gm. S 0.66 gm. N
5	0.328	0.152	0.022	0.154	3.46	134	
6	0.827	0.212	0.014	0.602	3.81	166	6.7 gm. cysteic acid as Na salt = { 1.123 gm. S 0.49 gm. N
7	0.298	0.085	0.006	0.207	3.33	144	
8	0.915	0.178	0.013	0.724	3.78	178	7.0 gm. cysteic acid as Na salt = { 1.171 gm. S 0.510 gm. N
9	0.342	0.075	0.010	0.256	3.28	105	
10	1.120	0.185	0.010	0.925	4.10	184	3.7 gm. isethionic acid* as Na salt = 0.795 gm. S. Slight diarrhea, also diuresis.
11	0.746	0.111	0.013	0.622	3.76	157	Soft stools.
12	0.637	0.134	0.014	0.489	3.44	141	
13	0.680	0.120	0.015	0.545	3.15	100	2.8 gm. isethionic acid* as Na salt = 0.613 gm. S.
14	0.359	0.131	0.012	0.215	3.33	94	
15	1.452	0.150	0.012	1.291	3.28	103	8.2 gm. isethionic acid† as Na salt = 1.766 gm. S. Inorganic S = 0.012 gm.
16	0.244	0.121	0.016	0.107	3.55	108	7.5 gm. cystine = { 2.00 gm. S 0.878 gm. N
17	1.371	0.698	0.016	0.656	4.06	301	
18	0.675	0.213	0.020	0.443	3.80	253	Soft stools.
19	0.298	0.185	0.013	0.100	3.36	90	
20	0.275	0.142	0.007	0.126	3.56	122	7 gm. taurocholic acid = { 0.441 gm. S 0.195 gm. N
21	0.244	0.142	0.009	0.094	3.74	106	Soft stools. Weight 15.9 kilos.

* Prepared from taurine by treatment with HNO_3.

† Kahlbaum preparation—50 per cent aqueous solution.

Daily Diet.

Cracker meal... 200 gm.

Crisco.. 40 "

Sucrose... 60 "

Fullers' earth.. 50 "

Condensed milk.. 100 cc.

Distilled water sufficient to make a "stiff dough."

TABLE II.

Experiment 2. Dog 2.

Day of experiment.	Total sulfur.	Inorganic sulfur.	Ethereal sulfur.	Neutral sulfur.	Total nitrogen.	α-amino nitrogen.	Remarks.
	gm.	gm.	gm.	gm.	gm.	mg.	
1	0.103	0.042	0.009	0.052	3.28	49	Weight 12.7 kilos.
2	0.103	0.053	0.009	0.041	3.11	60	
3	0.097	0.047	0.008	0.042	2.86	50	
4	0.919	0.752	0.005	0.163	3.30	109	7.5 gm. cystine = $\begin{cases}2.0 \text{ gm. S} \\ 0.878 \text{ gm. N}\end{cases}$
5	0.267	0.164	9.005	0.098	2.90	79	
6	0.147	0.077	0.010	0.061	2.55	77	
7	0.712	0.073	0.007	0.632	2.76	63	11 cc. of Kahlbaum's 50 per cent isethionic acid as Na salt = 1.035 gm. S.*
8	0.118	0.039	0.007	0.073	2.87	66	
9	0.079	0.023	0.009	0.048	2.54	50	
10	0.526	0.080	0.006	0.440	2.76	55	5.8 gm. cysteic acid as Na salt = $\begin{cases}0.989 \text{ gm. S} \\ 0.425 \text{ gm. N}\end{cases}$
11	0.189	0.133	0.010	0.046	2.64	67	
12	0.125	0.015	0.006	0.104	2.67	56	
13	1.203	0.051	0.005	1.147	3.03	515	6 gm. taurine = $\begin{cases}1.54 \text{ gm. S} \\ 0.67 \text{ gm. N}\end{cases}$
14	0.262	0.021	0.008	0.233	2.87	84	
15	0.146	0.036	0.007	0.102	2.52	76	
16	0.125	0.030	0.012	0.083	3.05	65	
17	0.111	0.028	0.006	0.077	2.50	68	
18	0.888	0.708	0.006	0.174	3.10	155	7.5 gm. cystine = $\begin{cases}2.0 \text{ gm. S} \\ 0.878 \text{ gm. N}\end{cases}$
19	0.317	0.134	0.017	0.166	2.60	95	
20	0.224	0.104	0.009	0.111	2.80	46	
21	0.152	0.048	0.034	0.070	2.80	68	

* Contains 0.013 gm. S as sulfate.

Diet.

Cracker meal.. 175 gm.
Crisco.. 25 "
Sucrose... 80 "
Fullers' earth.. 40 "
Condensed milk.. 90 cc.
20 per cent beef extract.............................. 10 "

to variability for it is difficult in experiments of several weeks duration to eliminate daily fluctuations in the output of urinary constituents and the selection of normal values is difficult. In the résumé the normal values were obtained either by taking the average of several days of the fore period or by using the figures preceding the experimental day.

TABLE III.

Experiment 3. Dog 2.*

Day of experiment.	Urinary sulfur.			Urinary nitrogen.		Remarks.
	Total.	Inorganic.	Neutral plus ethereal.	Total.	α-amino nitrogen.	
	gm.	gm.	gm.	gm.	mg.	
1	0.398	0.113	0.185			Weight 14.5 kilos.
2	0.247	0.106	0.141			
3	0.671	0.473	0.198			2 gm. Na₂S₂O₃·5H₂O = 0.516 gm. S†
4	0.179	0.082	0.097			
5	0.537	0.426†	0.111			1.5 gm. Na₂SO₃ = 0.381 gm. S‡§
6	0.149	0.071	0.078			
7	0.404	0.300	0.104			1 gm. Na₂S₂O₃·5H₂O = 0.258 gm. S‖
8	0.155	0.075	0.080	2.49	52	7.5 gm. cystine = { 2.0 gm. S
9	0.946	0.765	0.181	3.21	75	{ 0.878 gm. N
10	0.361	0.196	0.165	2.48	59	

* About a month elapsed between Experiments 2 and 3, during which time the dog had been returned to the animal house and received a meat and bread diet.

† When the urine was acidified with H_3PO_4 and distilled, S and SO_2 were obtained in large amounts.

‡ 0.046 gm. S was present as sulfate.

§ Distillation shows only traces of sulfites in the urine.

‖ Urine contains only a small amount of thiosulfate.

Diet.

Cracker meal	150 gm.
Crisco	20 "
Sucrose	65 "
Fullers' earth	40 "
Condensed milk	75 cc.
20 per cent beef extract	10 "

TABLE IV.

Experiment 4.   Dog 3.*

Day of experiment.	Urinary sulfur.			Total nitrogen.	Remarks.
	Total.	Inorganic plus ethereal.	Neutral.		
	gm.	*gm.*	*gm.*	*gm.*	
1	0.110	0.074	0.036	2.50	Weight 11.4 kilos.
2	0.126	0.083	0.043	2.68	
3	0.112	0.072	0.040	2.38	
4	1.084	0.799	0.285	2.77	7 gm. cystine = $\begin{cases} 0.819 \text{ gm. N} \\ 1.87 \text{ gm. S} \end{cases}$
5	0.241	0.186	0.055	2.02	
6	0.136	0.095	0.041	1.96	

* Young growing dog.

Diet.

Cracker meal.. 75 gm.

Crisco.. 15 "

Sucrose... 30 "

Fullers' earth.. 25 "

Condensed milk.. 50 cc.

TABLE V.

Experiment 5.*   Dog 4 (13.5 kilos).

Day of experiment.	Total sulfur.	Inorganic sulfur.	Ethereal sulfur.	Neutral sulfur.	Total nitrogen.	Remarks.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	
1	0.135	0.091	0.005	0.039	2.50	
2	0.164	0.104	0.009	0.051	2.51	
3	0.180	0.118	0.007	0.055	2.58	0.210 gm. S as purified taurocholic acid given by mouth.
4	0.168	0.101	0.004	0.063	2.42	
5	0.166	0.097	0.007	0.061	2.40	

* Experiments 5 and 6 were carried out in this laboratory by Mr. Thomas Watson.

Daily Diet.

Lard.. 30 gm.

Butter.. 10 "

Cracker meal.. 200 "

Sugar... 60 "

Milk.. 100 cc.

Kaolin.. 50 "

With cysteic acid there is an increase neither in urinary sulfates nor in amino nitrogen[1] which indicates that deamination took place but that the remainder of the molecule was apparently excreted unchanged. Neither sulfurous nor thiosulfuric acid was found in the urine. In contrast to these findings are the results which were obtained on feeding taurine. The amino nitrogen figures indicate that taurine does not combine with urea to form taurocarbamic acid and the sulfur values indicate that there is no appreciable oxidation of the sulfur. These facts agree with the previous findings of Schmidt and Allen on man (9). Cysteic acid, which contains an amino group in a position alpha

TABLE VI.

Experiment 6.*   Dog 4 (13.5 kilos).

Day of experiment.	Total sulfur.	Inorganic sulfur.	Ethereal sulfur.	Neutral sulfur.	Total nitrogen.	Remarks.
gm.	gm.	gm.	gm.	gm.	gm.	
1	0.175	0.095	0.012	0.068	2.70	
2	0.177	0.098	0.012	0.067	2.66	
3	0.171	0.096	0.009	0.066	2.62	
4	0.945	0.852	0.020	0.073	3.76	8.7 gm. cystine by mouth = $\begin{cases} 2.32 \text{ gm. S} \\ 1.01 \text{ gm. N} \end{cases}$
5	0.456	0.430	0.013	0.013	2.56	
6	0.187	0.098	0.011	0.078	2.58	
7	0.175	0.096	0.011	0.068	2.68	

* The daily diet was the same as in Experiment 5.

to the carboxyl group, is deaminized on passage through the body despite the fact that it also contains a sulfonic group, while taurine, which reacts *in vitro* in all respects like an α-amino-carboxylic acid, is not so deaminized. The replacement of the amino group of taurine by a hydroxyl group to give isethionic acid does not lead to an increase in urinary sulfates. A marked increase in the output of both sulfur and nitrogen coincident with the administration of the first dose of isethionic acid to Dog 1 was found. Apparently a factor other than the substance administered was responsible for the increase in endogenous metab-

[1] Cysteic acid yields its nitrogen quantitatively when treated with HNO_2.

TABLE VII.

Résumé Showing the Urinary Recovery of the Substances Ingested. *

Experiment 1.

Substance ingested.	Inorganic sulfur.			Neutral sulfur.			Total sulfur.			Total nitrogen.			α-amino nitrogen.		
	Experimental value.	Normal value.	Recovery.	Experimental value.	Normal value.	Recovery.	Experimental value.	Normal value.	Recovery.	Experimental value.	Normal value.	Recovery.	Experimental value.	Normal value.	Recovery.
	gm.	gm.	per cent	gm.	gm.	per cent	gm.	gm.	per cent	gm.	gm.	per cent	mg.	mg.	per cent
Taurine..........	No increase.			1.117	0.117	67	1.277	0.263	68	4.20	3.50	106	493	114	58
Cysteic acid 1.															
Output 1st day....	0.212	0.141	6	0.602	0.117	43	0.827	0.263	50	3.81	3.50	63	No increase.		
" 2nd "				0.207	0.117	8	0.298	0.263	3						
						51			53						
Cysteic acid 2.															
Output 1st day....	0.178	0.141	3	0.724	0.117	52	0.915	0.263	56	3.78	3.50	74	No increase.		
" 2nd "				0.256	0.117	12	0.342	0.263	6						
						64			62						
Isethionic acid 1.															
Output 1st day....				0.925	0.489	55	1.120	0.637	61†						
" 2nd "				0.622	0.489	17	0.746	0.637	14						
						72			75						

Isethionic acid 2..........			0.545	0.215	54	0.680	0.359	52							
" 3..........			1.291	0.107	61	1.452	0.244	68							
Cystine.															
Output 1st day..........	0.698	0.156	27	0.656	0.107	28	1.371	0.272	55	4.06	3.55	58	301	110	22
" 2nd "..........	0.213	0.156	3	0.443	0.107	17	0.675	0.272	20	3.80	3.55	28	253	110	16
			30			45			75			86			38
Taurocholic acid.§															
Output 1st day.‡..........	0.698	0.156	27				0.822	0.272	28	3.87	3.55	36			
" 2nd " .‡..........	0.213	0.156	3				0.339	0.272	3	3.66	3.55	12			
			30						31			48			
Experiment 2.															
Cystine.															
Output 1st day..........	0.752	0.047	35	0.163	0.045	6	0.919	0.101	41	3.30	2.90	46	109	53	6
" 2nd "..........	0.164	0.047	6	0.098	0.045	3	0.267	0.101	8	No increase.					
" 3rd "	0.077	0.047	2	0.061	0.045	1	0.147	0.101	2	"					
			43			10			51						

* Blank spaces indicate that the output values are within the limits of the normal daily variation.

† The abnormally high values for the output of nitrogen and sulfur were apparently due to factors other than the ingestion of isethionic acid. The sulfur values of the 2nd day following the ingestion of isethionic acid are taken as normal values for the purpose of calculating sulfur output.

‡ The calculations are on the basis of catabolized cystine.

§ The figures in Table I indicate that taurocholic acid is not eliminated in the urine.

TABLE VII—Concluded.

Experiment 2—Concluded.

Substance ingested	Inorganic sulfur			Neutral sulfur			Total sulfur			Total nitrogen			α-amino nitrogen		
	Experimental value	Normal value	Recovery	Experimental value	Normal value	Recovery	Experimental value	Normal value	Recovery	Experimental value	Normal value	Recovery	Experimental value	Normal value	Recovery
	gm.	gm.	per cent	gm.	gm.	per cent	gm.	gm.	per cent	gm.	gm.	per cent	mg.	mg.	per cent
Isethionic acid.															
Output 1st day				0.632	0.045	57	0.712	0.101	59						
" 2nd "				0.073	0.045	3	0.118	0.101	1						
						60			60						
Cysteic acid.															
Output 1st day	0.133	0.050	10	0.440	0.046	40	0.526	0.125	41	2.76	2.59	40	No increase		
" 2nd "							0.189	0.125	6						
						60			47						
Taurine.															
Output 1st day				1.147	0.102	68	1.203	0.146	69	3.03	2.70	49	515	76	66
" 2nd "				0.233	0.102	9	0.263	0.146	8	2.87	2.70	25			
						77			77			74			
Cystine.															
Output 1st day	0.708	0.048	33	0.174	0.070	5	0.888	0.152	34	3.10	2.60	57	155	68	10
" 2nd "	0.134	0.048	4	0.166	0.070	5	0.317	0.152	8				95	68	3
" 3rd "	0.104	0.048	3	0.111	0.070	2	0.224	0.152	4						
			40			12			46						13

Experiment 3.||

Sodium thiosulfate.	0.473	0.076	77	0.198	0.085	22	0.671	0.168	98			
" sulfite.	0.426	0.076	92	0.117	0.085	8	0.537	0.168	97			
" thiosulfate.	0.300	0.076	87	0.104	0.085	7	0.404	0.168	92			
Cystine.												
Output 1st day.	0.765	0.076	35	0.181	0.085	5	0.946	0.168	39	3.21	2.48	83
" 2nd "	0.196	0.076	6	0.165	0.085	4	0.361	0.168	10			
			41			9			49			

Experiment 4.¶

Cystine.												
Output 1st day.	0.799	0.076	39	0.285	0.041	13	1.084	0.116	52	2.77	2.20	70
" 2nd "	0.186	0.076	6				0.241	0.116	7			
			45						59			

Experiment 6.

Cystine.												
Output 1st day.	0.852	0.096	33				0.945	0.175	33	3.76	2.62	113
" 2nd "	0.430	0.096	14				0.456	0.175	12			
			47						45			

|| In this experiment the figures given in the neutral sulfur column are neutral plus ethereal sulfur.
¶ In this experiment the figures given in the inorganic sulfur column are for total sulfates.

olism since subsequent doses of the same substance to this animal and to another dog did not show the effect.

No appreciable amount of thiosulfuric acid was found in the urine following the ingestion of either cysteic acid, taurine, or isethionic acid. This raises the question why other experimenters found thiosulfates on feeding compounds containing sulfonic acid radicals. It is possible that bacterial action was a factor concerned in their experiments. The presence of small quantities of sulfurous or thiosulfuric acid in urine is easily determined by acidifying the urine with H_3PO_4 and distilling in an atmosphere of CO_2. Our experiments with sodium sulfite and sodium thiosulfate indicate that if such substances as isethionic acid and taurine are reduced to sulfurous acid and this compound is absorbed, oxidation is both complete and rapid.[2] If large quantities of thiosulfuric acid are formed from the administered substance by bacterial action and absorption subsequently takes place the urine of the animal will contain thiosulfuric acid, the amount depending on the dosage of thiosulfate which is absorbed. Our experiments with sulfites and thiosulfates are in agreement with the results of Salkowski (15), Lasch (16), and Rost and Franz (17), and show that moderate doses are readily oxidized to sulfates.

Administration of a 7.5 gm. dose of cystine to Dog 1 not only led to a considerable oxidation (30 per cent) of the amino-acid as seen from the increase in urinary sulfates, but curiously, about one-half of the amount administered was excreted in the urine unchanged, the elimination of cystine extending over a period of 2 days. The figures for both the neutral sulfur and the amino nitrogen support this statement and the presence of cystine in the urine was established by subsequent isolation with the aid of Gaskell's method (18). The elimination of unaltered cystine in the urine of Dog 1 was probably due to unusually rapid absorption but it is by no means clear why the elimination of free amino-acid extended over the 2nd day. Similar doses of cystine administered to Dogs 2 (Experiments 2 and 3), 3 (Experiment 4), and 4 (Experiment 6) were followed by minimal increases in neutral sulfur. The major portions of the absorbed cystine were oxidized as evidenced by a very marked increase in the elimination of urinary

[2] Unpublished investigations with sulfurous acid carried out by one of us (C. L. A. S.) on man gave similar results.

sulfates.   Ingestion of 7.5 gm. of cystine did not lead to the
appearance of either sulfurous or thiosulfuric acid in the urine of
the experimental animals.

From the results of Experiments 1, 2 (second dose), 3, 4, and 6
(see also résumé table) it will be noted that following the ingestion
of cystine the magnitude of the figures for nitrogen elimination is
larger than the magnitude of the corresponding sulfur figures.
From this list we may possibly eliminate the comparison of the
nitrogen and sulfur figures of Experiments 1, 2 (second dose),
and 4 as being not far outside of the limits of the normal variability
but the difference in the percentages of cystine nitrogen and sulfur
recovered in the urine as found in Experiments 3 and 4 cannot be
so eliminated.   If the difference is due to a specific demand on the
part of the animal for anabolic sulfur a retention of both nitrogen
and sulfur would be expected since sulfur is built into the protein
molecule in the form of cystine.   It is possible that we are dealing
with a phenomenon analogous to that studied by Folin and
Denis (19).   They found that as a result of bacterial action on the
nitrogenous material which has passed into the large intestines the
ammonia content of the portal blood is increased.   If cystine is
decomposed by bacterial action to NH_3 and H_2S the former can
by absorption enter the blood stream while the latter may be
eliminated through the intestines.

Our results also show that the elimination of nitrogen after
ingestion of cystine precedes that of sulfur.   This fact is shown
in Experiments 2, 3, 4, and 6.   Certain of the figures for the output
of sulfur on the 2nd and 3rd day after ingestion of cystine prob-
ably lie within the variability of the normal value; all, however,
point in the same direction.   The lag in the excretion of sulfur is
apparently not due to a lag in the elimination of sulfates since the
experiment with Na_2SO_3 indicates very prompt elimination.   It
appears to us more reasonable to assume that deamination of the
cystine precedes the oxidation of sulfur, with the result that the
elimination of sulfur lags behind the nitrogen.   This explanation
is in accord with the idea of Lewis (20) that the breaking up of the
cystine molecule is dependent upon and takes place subsequent
to deamination.

A negative Pettenkofer test for bile acids and normal values
for urinary sulfur and nitrogen were obtained following the in-

gestion of taurocholic acid and indicate that the urine is not a channel for the elimination of bile acids. Even in icterus the amount of bile acids eliminated in the urine per 24 hours does not exceed several hundred milligrams.[3]

SUMMARY.

Experiments were carried out on dogs to determine the fate of certain sulfur compounds with especial reference to those concerned in bile metabolsim.

It was found that taurine is excreted in the urine unchanged and not as taurocarbamic acid as stated by Salkowski. Cysteic acid is deaminized, but the remainder of the molecule is excreted in the urine unchanged. Administration of isethionic acid is not followed by an increase in urinary sulfates as stated by several investigators. The urine is not a channel for the elimination of bile acids when the latter are fed. When given in large doses the major portion of the absorbed cystine is oxidized to sulfates which are eliminated in the urine. With the exception of large doses of sodium thiosulfate the ingestion of the various sulfur compounds under consideration did not lead to the appearance of appreciable amounts of sulfurous or thiosulfuric acid in the urine.

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[3] Unpublished investigations of Schmidt and Merrill.

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THE ACETONURIA OF DIABETES.*

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Both Shaffer (1921) and Woodyatt (1921), in recent articles, have discussed the acetonuria found in diabetes, in starvation, and in abnormal nutrition due to diets low in carbohydrate as if they could be attributed to the same type of metabolic disturbance. This seems to be the conception of a majority of the writers on the subject at the present time, although some do not accept this theory.[1] The outstanding difference between the two conditions is that normal subjects develop acetonuria when deprived of food, while diabetic patients often show less acetonuria during a fast than they did before. Labbé, Labbé, and Nepveux (1921) have explained this phenomenon as follows: Diabetics often receive more food, particularly more fat, than they can burn, and this excess of fat leads to the production of acetone bodies; when this excess of fat is no longer fed, the excretion of the acetone bodies decreases; normal subjects, on the other hand, receive more carbohydrate than they need to burn the fat taken, and the withdrawal of this carbohydrate causes a production of acetone bodies from fat—in the case of complete inanition from body fat—and so apparently we get an entirely different response to starvation from that given by diabetics. This explanation is similar to that suggested by the papers of Shaffer and Woodyatt cited above. Ladd and Palmer (1920–21) have shown that diets which cause moderate increases of acetone excretion by diabetic patients are essentially similar to those reported by Zeller (1914) and Lang (1915) as

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* The subject matter presented here formed part of a paper read before the American Society of Biological Chemists in December, 1921 (Hubbard, Nicholson, and Wright, 1922).

[1] See Joslin (1917), p. 162.

causing similar increases in normal subjects. In the paper
presented here further evidence is given to support the work of
Ladd and Palmer, and a method for studying differences in the
response of the subject to ingested fat and to fat drawn from the
reserves of the body is suggested.

In a former paper from this institution (Hubbard and Wright,
1922) on the study of the acetonuria developed by normal sub-
jects fed a diet high in fat and low in carbohydrate, the following
formula was proposed for expressing the ketogenic balance of any
diet as a molecular ratio:

$$100 \times \frac{1.5 \text{ (weight carbohydrate} + 25 \text{ per cent weight protein)}}{95 \text{ per cent weight fat}}$$

In this formula, which is based on the assumption that 1 molecule
of glucose will burn 1 molecule of ketogenic material,[2] the factor
1.5 represents the figure by which glucose must be multiplied to
give the molecular equivalent of fatty acid, 25 per cent weight
protein represents the probable amount of glucose which can be
derived from protein above that needed to burn the acetone bodies
which are derived from the same protein, and 95 per cent weight
fat represents the amount of fatty acids which fat yields on hydrol-
ysis.   From a study of the acetone found in the urine as compared
with a series of diets expressed in terms of this ratio it was con-
cluded that the value of the ratio was about 80 per cent when the
acetone excretion became normal.   Traces of acetone were found
in some cases when the ratio had a higher value than this, but
the figure given seemed to represent the value of the ratio of "the
border-line diet" as accurately as it was possible to determine it.
This "border-line diet" has the same composition as that described
by Woodyatt (1921):

$$2 \times \text{carbohydrate} + \tfrac{1}{2} \text{ protein} = \text{fat}$$

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[2] Shaffer, in a paper recently read before the American Society of Biolog-
ical Chemists, has stated that the ratio of 1 molecule of glucose to 2 of
fatty acid represents the conditions for complete oxidation of these com-
pounds in the body, rather than a ratio of 1 molecule to 1 molecule on
which Woodyatt's formula and the one in this paper are based.   He stated,
however, that a ratio of 1 molecule to 1 molecule gives a margin of safety
which makes it valuable in controlling the actual diets of diabetic patients
(Shaffer, 1922).

It was necessary to introduce certain changes into the expression of the ratio before it could be applied to the study of cases of diabetes. Most of the patients, for at least a part of the time during which they were under observation, received diets which did not maintain metabolic equilibrium. To allow for this fact the following plan was adopted: it was assumed that all the calories not furnished by retained (ingested and not excreted) carbohydrate, or by metabolized protein, were furnished by fat, either by fat taken in the diet or drawn from the reserve supplies of the body; this assumption may not be strictly correct, but it must be nearly so in the case of most diabetic patients, because their supply of glycogen available for combustion has been depleted by the disease. It was impossible to determine exactly how much fat a patient must draw from his reserves in a day, and the following calculation was used to estimate the amount roughly: the basal metabolism was determined, or, in most cases, calculated from the metabolism of normal subjects of the same height and weight, and an excess of 20 per cent over this was figured as the probable requirement of a diabetic patient under treatment, who was taking the mildest possible form of exercise. It has been found that such diets would maintain the body weight of normal subjects under similar conditions (Hubbard and Wright, 1922), and, while some diabetics maintain their weight on a smaller intake than this, the figure, 120 per cent of the basal requirement, seemed as good a first approximation as could be made for the purpose. The calories from carbohydrate utilized by the organism were calculated by multiplying the difference between the carbohydrate intake and the glucose excreted in the urine by 4; the calories derived from protein can be calculated by multiplying the total nitrogen in the urine by 6.25 to convert it into terms of protein, and then multiplying the product by 4 to convert it into calories, or they can be calculated directly from the protein fed if the subject is in nitrogen equilibrium. The sum of the calories derived from carbohydrate and from protein was subtracted from the estimated caloric requirement to give the calories derived from fat, and this difference was divided by 9 to convert it into terms of grams of fat burned by the patient. The mathematical expression for the weight of total fat burned based on these assumptions and calculations is:

$$\frac{120 \text{ per cent calories} - 4 \text{ (gm. carbohydrate fed} - \text{gm. urine sugar)} - 25 \times \text{gm. urine N}}{9} = \text{gm. fat}$$

In the expression

$$100 \times \frac{1.5 \text{ (weight carbohydrate} + 25 \text{ per cent weight protein)}}{95 \text{ per cent weight fat}}$$

the numerator, which represents the antiketogenic material expressed as glucose, must be changed for studying diabetic patients. When glucose is present in the urine, the amount found should be subtracted from the carbohydrate ingested (Shaffer, 1921); when the patient is not approximately in nitrogen equilibrium, the urinary nitrogen should be multiplied by 1.6 $\left(1.6 = \dfrac{6.25}{4}\right)$ to give the amount of glucose derived from the protein burned greater than that which is necessary for complete combustion of the ketogenic material from the same protein. The mathematical formula for expressing the ketogenic balance of a diet fed to a diabetic similar to the one used in studying the diets of normal subjects (Hubbard and Wright, 1922) is:

$$100 \times \frac{1.5 \text{ (weight carbohydrate} - \text{weight urine sugar} + 1.6 \text{ weight urine N)}}{95 \text{ per cent weight fat}}$$

The amount of fat should be estimated in some such way as that given above, or, if possible, the proportions of the different foodstuffs should be calculated from complete respiratory exchange data.

Seven cases have been selected out of twenty-five studied to illustrate the method. All the patients were receiving a treatment similar to that outlined by Allen (1913) and Joslin (1917) and we believe that the cases selected for presentation took only the food furnished them from the diet kitchen. After a short period of starvation, or, more often, of marked undernutrition, the food furnished was increased slowly. Protein was the food first increased, and the patients were receiving enough of this foodstuff to maintain them in nitrogen equilibrium during the larger part of the time during which they were studied and all were receiving enough of this foodstuff for this purpose when the acetone excretion became normal. This made it possible to base the calcula-

tion on the protein intake without introducing errors in excess
of the experimental ones, and made the results more directly com-
parable with those previously published (Hubbard and Wright,
1922). None of the subjects showed temperatures above normal
during the study. Included in each case report is a table in which
the diet, weight, alveolar carbon dioxide tension, blood sugar, and
the excretion of sugar and of the acetone bodies in the urine is
recorded. The alveolar carbon dioxide tension was determined
by the method of Fridericia (Fridericia, 1914; Poulton, 1915);
blood sugar by the Benedict (1918) modification of the method of
Lewis and Benedict (1915); and the acetone bodies by a method
recently published (Hubbard, 1921).

A chart has been plotted for each case presented which shows
the relationship of the calories fed to the calculated basal caloric
requirement of a normal subject of the same height and weight;[3]
changes in body weight; the values of the ratios described above
based on the fat taken in the diet and on the probable amount of
fat actually burned by the patient; and the total acetone bodies
found in the urine expressed as acetone. In these charts a cross
has been used to designate a positive sodium nitroprusside test
at certain times when quantitative analyses were not made.

Case 1 had a mild type of diabetes which had lasted only a short time.
A study of the chart shows that 120 per cent of the normal basal require-
ment represented approximately the caloric needs during the period. The
value of the ratio glucose to fat burned appeared to be about 90 per cent
when the first slight increases of acetone excretion occurred. There was
a slight increase in the amount of acetone excreted on Aug. 19 when the fat
intake was increased, but no change was made in the amounts of protein
and carbohydrate in the diet. This observation seems to show that fat
fed as distinct from body fat, may have an effect upon the excretion of
acetone. This finding is in harmony with the findings and theories stated
by Allen, Stillman, and Fitz.[4] Slight temporary increases in the excretion
of acetone were found at other times.

Case 2 had a mild type of the disease which had lasted only for a short
time. Chart 2 shows that 120 per cent of her calculated basal metabolism
represented approximately the caloric needs during the period of study.
The amount of acetone found in the urine was very small when the ratio
glucose to fat burned had a value of 100 per cent, and finally decreased to

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[3] The tables given in Lusk's book (Lusk, 1917), pp. 126 to 129, were used
in making these calculations.

[4] Allen, Stillman, and Fitz (1919), Chapter VI, p. 500.

TABLE I.

Case 1.

	Diet.					Alveolar CO₂.	Blood sugar.	Urine.				
Date.	Carbohydrate.	Protein.	Fat.	Calories.	Weight.	Alveolar CO_2.	Blood sugar.	Volume.	Sugar.		Acetone.	β-hydroxybutyric acid.
1921	gm.	gm.	gm.		kg.	mm.	per cent	cc.	per cent	gm.	gm.	gm.
July 19	?	?	?	?	56 7			1,850	5.2	96.2	0.109	
" 20	59	30	3	383			0.390	1,640	4.2	68.8	0.145	
" 21	64	33	0	388				1,260	3.1	39.0	0.274	0.469
" 22	64	33	0	388				1,120	3.5	39.9	0.206	0.374
" 23	64	33	0	388				870	2.3	19.7		
" 24	64	33	0	388		33		850	2.0	17.0		
" 25	64	33	0	388	56 2			640	1.2	8.0		
" 26	64	33	0	388				520	0.3	1.4	0.057	0.051
" 27	64	33	0	388				520	0.5	2.6	0.040	0.053
" 28	64	33	0	388	55 8			760	0.4	2.8	0.072	0.090
" 29	0	0	0	0				600	0	0		
" 30	3	2	0	20		40		1,420	0	0		
" 31	8	10	6	126	55 6			1,370	0	0		
Aug. 1	8	12	6	134		34		1,510	0	0	0.719	2.12
" 2	10	15	10	190				1,300	0	0	0.586	2.02
" 3	12	20	15	263	54 3	31		1,280	0	0	0.925	2.37
" 4	15	26	25	389				1,040	0	0	0.751	1.59
" 5	20	29	29	457				1,100	0	0	0.534	1.08
" 6	20	29	29	457				1,260	0	0		
" 7	20	29	30	466	53 5	31	0.120	830	0	0		
" 8	25	40	40	620		31		940	0	0	0.125	0.713
" 9	25	48	50	742				1,120	0	0	0.353	0.658
" 10	30	50	50	770	54 3			1,500	0	0	0.328	0.563
" 11	35	58	65	957				940	0	0	0.266	0.390
" 12	40	71	70	1,074				1,070	0	0	0.368	0.433
" 13	40	71	70	1,074				1,150	0	0		
" 14	40	70	70	1,070	54 6		0.150	1,240	0	0		
" 15	40	70	70	1,070				960	0	0		
" 16	46	70	71	1,103				1,480	0	0	0.123	0.087
" 17	40	69	75	1,111	53 7			970	0	0	0.073	0.067
" 18	40	69	85	1,201				1,560	0	0	0.125	0.121
" 19	40	70	85	1,205		32		1,260	0	0	0.189	0.370
" 20	40	76	85	1,229				1,280	0	0	0.248	0.113
" 21	40	75	90	1,270	53 8			1,310	0	0		
" 22	40	75	90	1,270				1,380	0	0	0.072	0.065

TABLE I—*Concluded.*

Date.	Diet.				Weight.	Alveolar CO₂.	Blood sugar.	Urine.				
	Carbohydrate.	Protein.	Fat.	Calories.				Volume.	Sugar.		Acetone.	β-hydroxybutyric acid.
1921	*gm.*	*gm.*	*gm.*		*kg.*	*mm.*	*per cent*	*cc.*	*per cent*	*gm.*	*gm.*	*gm.*
Aug. 23	40	77	90	1,278				1,260	0	0	0.116	0.099
" 24	40	75	100	1,360	53.7		0.140	1,200	0	0	0.185	0.230
" 25	45	75	100	1,380				1,010	0	0	Positive.	
" 26	47	75	95	1,343				1,360	0	0	"	
" 27	50	75	100	1,400				1,160	0	0	"	
" 28	55	77	100	1,419	53.5			1,090	0	0	"	
" 29	55	75	100	1,420				1,200	0	0	Negative.	
" 30	55	76	100	1,424			0.150	1,010	0	0	"	
" 31	55	72	99	1,399	53.5			800	0	0	"	
Sept. 1	55	75	100	1,420				1,300	0	0	"	
" 2	55	75	100	1,420				1,050	0	0	"	
" 3	55	75	100	1,420				1,000	0	0	"	
" 4	55	75	100	1,420	53.0			800	0	0	"	
" 5	55	75	100	1,420				1,080	0	0	"	
" 6	55	75	100	1,420				1,060	0	0	"	
" 7	60	76	105	1,485	53.1			1,260	0	0	"	
" 8	60	76	105	1,489				1,080	0	0	"	
" 9	60	76	104	1,480				1,500	0	0	Positive.	
" 10	60	75	105	1,485			0.140	1,250	0	0	Negative.	
" 11	60	60	102	1,398	53.3			1,110	0	0	Positive.	
" 12	60	75	104	1,478				1,060	0	0	Negative.	
" 13	60	76	105	1,489				1,200	0	0	"	
" 14	60	52	85	1,213	53.3			1,460	0	0	"	
" 15	60	76	104	1,480				1,220	0	0	"	
" 16	54	64	81	1,201				1,340	0	0	Positive.	
" 17	60	76	105	1,489				1,180	0	0	"	
" 18	60	75	105	1,485	52.8			1,030	0	0	Negative.	
" 19	60	75	105	1,485				1,360	0	0	"	
" 20	60	74	104	1,472				1,180	0	0	Positive.	
" 21	60	75	105	1,485	52.8							

Results of the test with sodium nitroprusside and ammonia are given for specimens on which quantitative determinations of the acetone bodies were not made.

Under acetone is listed acetone plus diacetic acid.

Results of all acetone bodies are expressed in terms of acetone.
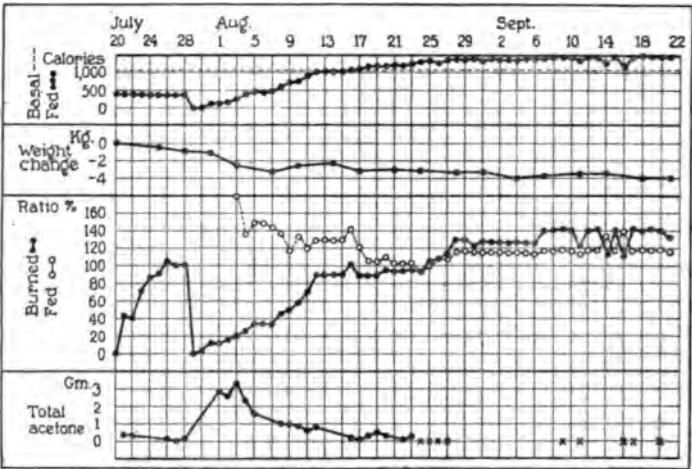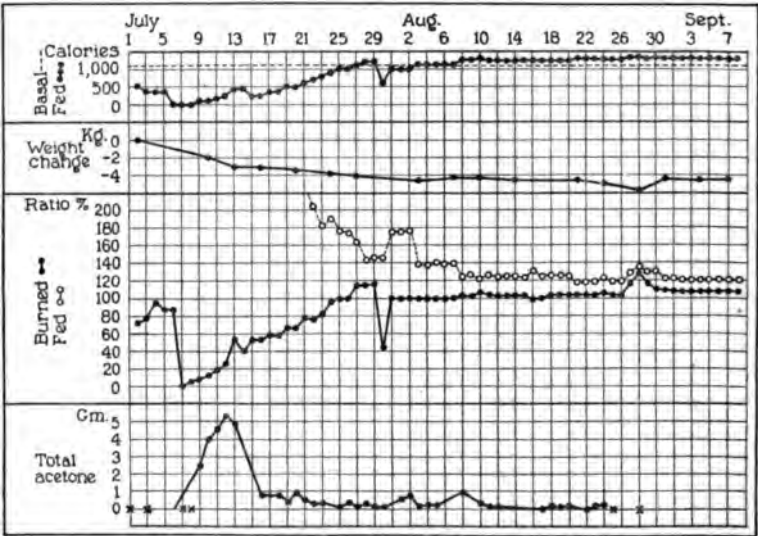
CHART 1. Case 1.

CHART 2. Case 2.

TABLE II.

Case 2.

Date	Carbohydrate	Protein	Fat	Calories	Weight	Alveolar CO₂	Blood sugar	Volume	Sugar		Acetone	β-hydroxybutyric acid
1921	*gm.*	*gm.*	*gm.*		*kg.*	*mm.*	*per cent*	*cc.*	*per cent*	*gm.*	*gm.*	*gm.*
July 1	?	?	?	?					2.5		Positive.	
" 2	55	81	63	1,111	54.1		0.225	1,560	1.6	24.9	Negative.	
" 3	64	33	18	550				1,320	1.0	13.0	Positive.	
" 4	64	33	0	388				1,100	0.3	3.0	Negative.	
" 5	64	33	0	388		36		840	0.8	6.9	"	
" 6	64	33	0	388				1,030	0.7	7.2	"	
" 7	0	0	0	0				710	Trace.		Positive.	
" 8	5	3	0	32				520	0	0	"	
" 9	5	9	6	110				720	0	0	0.438	1.95
" 10	8	10	6	126	52.1	33		1,050	0	0	0.776	3.26
" 11	10	20	6	174		32		830	0	0	0.812	3.78
" 12	15	26	11	263				1,010	0	0	1.03	4.36
" 13	33	36	18	438	51.1	29		780	Trace.		1.56	3.30
" 14	23	40	20	432		33		1,480	0	0		
" 15	36	27	0	252		36		1,325	0	0		0.587
" 16	36	27	0	252	50.9	36		1,700	0	0	0.312	0.467
" 17	36	36	10	378		36		1,720	0	0		0.428
" 18	36	36	10	378				1,410	0	0	0.368	0.428
" 19	40	41	20	504		33		1,225	0	0	0.184	0.216
" 20	40	41	20	504	50.7			1,680	0	0	0.616	0.264
" 21	39	40	30	594		33		1,380	0	0	0.230	0.286
" 22	40	45	40	700		34	0.150	1,270	0	0	0.180	0.174
" 23	45	50	50	820				1,560	0	0	0.161	0.168
" 24	50	60	54	926	50.5	34		1,610	0	0		
" 25	50	65	59	991				1,220	0	0	0.026	0.060
" 26	50	65	60	1,000				1,320	0	0	0.168	0.100
" 27	55	70	70	1,130	50.3			1,040	0	0	0.077	0.093
" 28	55	70	80	1,220			0.170	950	0.3	2.9	0.142	0.111
" 29	56	70	80	1,224				1,425	Trace.		0.133	0.040
" 30	27	37	40	616				1,300	0	0	0.073	0.051
" 31	50	65	60	1,000				1,660	0	0		
Aug. 1	50	65	60	1,000		38		1,160	0	0	0.318	0.214
" 2	50	65	59	991				1,200	0	0	0.177	0.486
" 3	50	65	75	1,135	49.5			1,500	0	0	0.122	0.077
" 4	50	65	76	1,144				1,300	0	0	0.128	0.100
" 5	50	65	74	1,120				1,100	0	0	0.105	0.050
" 6	50	65	75	1,135				1,500	0	0		
" 7	50	65	75	1,135	49.8	39	0.150	1,480	0	0		

TABLE II—*Concluded*.

Date	Carbohydrate.	Protein.	Fat.	Calories.	Weight.	Alveolar CO₂.	Blood sugar.	Volume.	Sugar. (per cent)	Sugar. (gm.)	Acetone.	β-hydroxybutyric acid.
1921	gm.	gm.	gm.		kg.	mm.	per cent	cc.	per cent	gm.	gm.	gm.
Aug. 8	50	69	85	1,241				1,600	0	0	0.346	0.566
" 9	50	69	84	1,241				1,200	0	0		
" 10	51	71	89	1,289	49.9			1,120	0	0	0.205	0.088
" 11	50	71	85	1,249			0.170	1,440	0	0	0.093	0.061
" 12	50	70	85	1,245				1,200	0	0	0.080	0.071
" 13	50	70	85	1,245				1,300	0	0		
" 14	50	70	85	1,245	49.5			1,450	0	0		
" 15	50	70	85	1,245				1,300	0	0		
" 16	52	69	84	1,240				1,400	0.2	2.8		
" 17	50	70	85	1,245	49.5			1,280	Trace.		0.042	0.037
" 18	50	70	85	1,245				1,320	0	0	0.077	0.063
" 19	50	70	85	1,245				1,290	0	0	0.076	0.057
" 20	50	70	85	1,245				1,170	0	0	0.066	0.062
" 21	50	69	90	1,286	49.6			1,250	0	0		
" 22	50	70	90	1,290				1,320	0	0	0.036	0.026
" 23	50	69	90	1,286				1,620	0	0	0.073	0.054
" 24	51	70	90	1,290	49.3			1,280	0	0	0.101	0.100
" 25	50	70	90	1,286				1,360	0	0	Positive.	
" 26	50	70	90	1,286				1,200	0	0	Negative.	
" 27	55	75	90	1,330				1,280	0	0	"	
" 28	59	75	90	1,350	48.4			1,320	0	0	Positive.	
" 29	55	75	90	1,330				1,180	0	0	Negative.	
" 30	55	74	90	1,326			0.190	1,290	0.3	3.3	"	
" 31	50	76	90	1,314	49.7			1,200	0	0	"	
Sept. 1	50	76	90	1,314				1,240	0	0	"	
" 2	50	75	90	1,310				1,100	0	0	"	
" 3	50	75	90	1,310				1,000	Trace.		"	
" 4	50	75	90	1,310	49.3			1,580	0	0	"	
" 5	50	75	90	1,310				1,340	0	0	"	
" 6	50	75	90	1,310				1,100	0	0	"	
" 7	50	75	90	1,310	49.4			1,100	0	0	"	
" 8	50	75	90	1,310				1,250	0	0	"	

Under "acetone" the results from the determinations of acetone plus acetoacetic acid are listed.

Results of the determinations on the acetone bodies are expressed in terms of acetone.

Results of tests with sodium nitroprusside and ammonia are given for specimens on which quantitative determinations of the acetone bodies were not made.

TABLE III.
Case 3.

Date.	Diet.				Weight.	Alveolar CO₂.	Blood sugar.	Urine.			Acetone.	β-hydroxybu-tyric acid.
	Carbohydrate.	Protein.	Fat.	Calories.				Volume.	Sugar.			
1921	gm.	gm.	gm.		kg.	mm.	per cent	cc.	per cent	gm.	gm.	gm.
June 28							0.295	920	6.3	57.9	Positive.	
" 29	50	85	90	1,350	67.4			1,050	2.8	29.0	"	
" 30	50	85	90	1,350				1,340	1.1	14.7	"	
July 1	52	88	88	1,352				1,300	1.0	13.0	"	
" 2	25	42	45	673				1,600	0.3	4.8	"	
" 3	25	42	45	673	67.2			1,100	0	0	"	
" 4	25	42	45	673				700	0	0	"	
" 5	30	50	50	770				980	0	0	"	
" 6	30	50	55	815	67.2	36		650	0	0	"	
" 7	30	55	60	880			0.165	1,220	0	0	0.500	1.76
" 8	35	65	65	985				720	0	0	Positive.	
" 9	35	65	70	1,050				460	0	0	0.525	1.12
" 10	41	66	71	1,067	66.8	30		1,040	0	0	0.126	0.171
" 11	55	70	80	1,220		30		750	0	0	0.129	0.207
" 12	102	58	19	811		31		1,300	0	0	0.126	0.117
" 13	102	58	0	640	66.6	36		900	0	0	0.028	0.022
" 14	86	56	0	568		36		875	0	0		
" 15	80	71	19	775	66.1	33		1,450	0	0	Negative.	
" 16	81	75	29	885				1,100	0	0	0.027	0.076
" 17	85	75	40	980		37		1,700	0	0	Negative.	
" 18	85	76	40	1,004				1,775	0	0	0.085	0.066
" 19	85	74	40	996				1,860	0	0	0.059	0.049
" 20	94	74	50	1,106	65.0		0.140	1,720	0	0	0.116	0.059
" 21	90	81	60	1,224				900	0	0	0.040	0.033
" 22	90	80	70	1,314				1,700	0	0	0.042	0.057
" 23	90	76	73	1,321		38		850	0	0	0.028	0.042
" 24	95	79	89	1,487	65.8			1,820	0	0	Negative.	
" 25	95	79	100	1,596				1,170	0	0	0.128	0.133
" 26	100	84	110	1,726				1,500	0	0	0.158	0.193
" 27	100	85	110	1,730	65.7		0.130	720	0	0	0.066	0.055
" 28	100	85	110	1,730				1,180	0	0	0.107	0.333
" 29	100	84	110	1,726				1,780	0	0	0.149	0.153
" 30	100	84	110	1,726				1,300	0	0	0.032	0.038
" 31	110	92	124	1,924	65.8			1,800	0	0	Negative.	
Aug. 1	110	90	125	1,925				1,000	0	0	0.003	0.043
" 2	110	90	125	1,925				990	0	0	0.015	0.030
" 3	110	74	115	1,771	65.5			990	0	0	Negative.	

Under "acetone" the results of the determinations of acetone plus acetoacetic acid are listed.

Results of the determinations of the acetone bodies are expressed in terms of acetone.

Results of tests with sodium nitroprusside and ammonia are given on specimens on which quantitative determinations of acetone were not made.

normal amounts when diets which gave this value had been ingested for about a month. The amount of fat fed seemed to increase the excretion of acetone independently of the calculated "amount burned" as it did in the first experiment on Aug. 8, and also apparently, on Aug. 1 and 2.

Case 3 had diabetes of a mild type which had lasted only for a short time. The caloric needs of this patient appear, from a study of weight changes, to have been a little less than 120 per cent of the caloric requirement. The acetone excretion was only slightly more than normal (less than 0.1 gm. per day) when the ratio had a value of 110 per cent. The influence of increases in fat fed as distinct from total fat burned was clearly shown in this case (July 25 to 30).
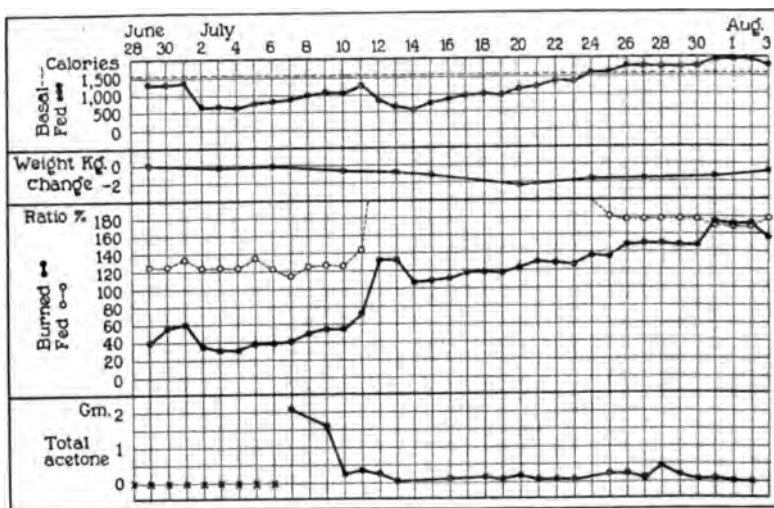
CHART 3. Case 3.

Case 4 had diabetes of a mild type which was of 15 years duration. The chart shows that 120 per cent of the basal requirement represented approximately the caloric needs under the conditions of study. A ratio of glucose to fat burned, which gave a value of 90 per cent, caused an excretion of approximately 0.5 gm. of acetone, while a ratio, which gave a value of 100 per cent, caused a normal excretion. The case shows that a marked lowering of the ratio glucose to fat burned caused an increased excretion of acetone when the ratio glucose to fat fed remained unchanged. Such a relative change in the values of the ratios follows proportional reduction of the three foodstuffs.

Case 5 had a rather mild form of diabetes which had lasted for 11 years. He was confined to his bed subsequent to an operation for gangrene when the study presented here was made. His weight stayed practically con-

TABLE IV.
Case 4.

Date.	Diet.				Weight.	Alveolar CO₂.	Blood sugar.	Urine.			Acetone.	β-hydroxybutyric acid.
	Carbohydrate.	Protein.	Fat.	Calories.				Volume.	Sugar.			
1921	gm.	gm.	gm.		kg.	mm.	per cent	cc.	per cent	gm.	gm.	gm.
Aug. 7	?	?	?	?	56.0			1,260	0	0	Positive.	
" 8	46	88	71	1,175				1,090	0	0	"	
" 9	45	83	90	1,322								
" 10	45	85	90	1,330				1,325	0	0	Negative.	
" 11	45	86	90	1,334	55.5			1,180	0	0	Positive.	
" 12	45	86	90	1,334			0.200	1,340	0	0	Negative.	
" 13	45	86	100	1,424				1,350	0	0	Positive.	
" 14	45	87	109	1,509	55.4			1,460	0	0	"	
" 15	48	87	129	1,701				1,470	0	0	0.366	0.190
" 16	45	86	110	1,514				1,440	0	0	0.144	0.141
" 17	45	85	110	1,510	55.7			1,320	0	0	0.187	0.246
" 18	45	89	110	1,530				1,530	0	0	0.193	0.226
" 19	45	85	110	1,510				1,510	0	0	0.236	0.378
" 20	45	85	110	1,510				1,440	0	0	0.144	0.396
" 21	50	85	109	1,521	56.2	34		1,360	0	0	Positive.	
" 22	50	85	110	1,530				1,400	0	0	0.199	0.165
" 23	50	85	110	1,530				1,420	0	0	0.130	0.085
" 24	50	85	110	1,530	56.0			1,360	0	0	0.103	0.093
" 25	50	85	111	1,539				1,740	0	0	Negative.	
" 26	50	85	110	1,530				1,460	0	0	"	
" 27	55	85	105	1,505				1,650	0	0	"	
" 28	60	85	100	1,480	56.0			1,560	0	0	"	
" 29	60	85	100	1,480				1,400	0	0	"	
" 30	60	86	100	1,484			0.210	1,200	0	0	"	
" 31	59	87	100	1,484	56.3			810	0	0	"	
Sept. 1	60	87	100	1,488				1,340	0	0	"	
" 2	60	85	100	1,480				580	0	0	"	
" 3	60	85	100	1,480				980	0	0	"	
" 4	60	84	100	1,476	55.1			1,030	0	0	"	
" 5	60	85	100	1,480				1,090	0	0		
" 6	60	85	100	1,480				1,740	0	0	Negative.	
" 7	60	86	100	1,484	55.6			1,720	0	0	"	
" 8	60	84	100	1,476				1,340	0	0	"	
" 9	60	85	100	1,480				1,530	0	0	"	
" 10	37	55	67	971				1,240	0	0	Positive.	

TABLE IV—*Concluded.*

Date.	Diet.				Weight.	Alveolar CO_2.	Blood sugar.	Urine.				β-hydroxybutyric acid.
	Carbohydrate.	Protein.	Fat.	Calories.				Volume.	Sugar.		Acetone.	
1921	gm.	gm.	gm.		kg.	mm.	per cent	cc.	per cent	gm.	gm.	gm.
Sept. 11	60	73	100	1,432	56.7			1,900	0	0	Negative.	
" 12	60	85	100	1,480				1,500	0	0	"	
" 13	60	84	100	1,476				1,620	0	0	"	
" 14	60	85	100	1,480	55.7			1,740	0	0	"	
" 15	60	86	100	1,484				1,930	0	0	"	
" 16	60	86	100	1,484				1,900	0	0	"	
" 17	60	86	100	1,484				1,600	0	0	"	
" 18	60	85	100	1,480	55.7			1,460	0	0	"	
" 19	60	85	100	1,480								

Under "acetone" the results from the determinations of acetone plus acetoacetic acid are listed.

Results of the determinations on the acetone bodies are expressed in terms of acetone.

Results of the test with sodium nitroprusside and ammonia are given on specimens on which quantitative determinations of the acetone bodies were not made.
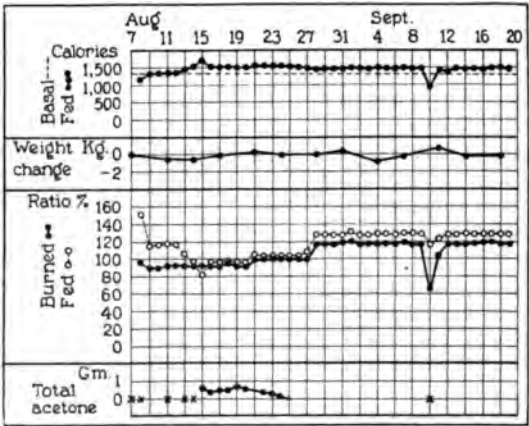
CHART 4. Case 4.

TABLE V.

Case 5.

	Diet.							Urine.			
Date.	Carbohydrate.	Protein.	Fat.	Calories.	Weight.	Alveolar CO₂.	Blood sugar.	Volume.	Sugar.	Acetone.	β-hydroxybutyric acid.
1921	gm.	gm.	gm.		kg.	mm.	per cent	cc.	per cent	gm.	gm.
July 1	30	70	70	1,030				2,150	0	0	Positive.	
" 2	30	70	70	1,030				1,340	0	0	"	
" 3	30	70	70	1,030	45.2			880	0	0	"	
" 4	30	70	70	1,030				750	0	0	Negative.	
" 5	30	70	70	1,030				1,000	0	0	Positive.	
" 6	30	70	80	1,120	45.6			600	0	0	"	
" 7	30	70	80	1,120			0.170	850	0	0	0.372	0.390
" 8	30	70	80	1,120				960	0	0	Positive.	
" 9	30	71	80	1,124		37		910	0	0	0.209	0.194
" 10	30	70	87	1,183	45.8			1,000	0	0	Positive.	
" 11	30	70	80	1,120				1,130	0	0	0.133	0.076
" 12	30	70	80	1,120				1,375	0	0	Positive.	0.146
" 13	30	70	80	1,120				945	0	0	"	0.087
" 14	29	71	80	1,120				580	0	0	"	
" 15	29	70	80	1,116				725	0	0	0.256	0.130
" 16	30	70	80	1,120	45.6			1,540	0	0	0.308	0.181
" 17	30	70	80	1,120		36	0.150	1,480	0	0	Positive.	
" 18	35	69	79	1,127				1,150	0	0	0.096	0.090
" 19	35	69	80	1,136				1,590	0	0	0.119	0.062
" 20	35	69	80	1,136	45.3			1,330	0	0	0.244	0.183
" 21	35	76	80	1,164				1,360	0	0	0.173	0.294
" 22	35	75	85	1,205			0.140	1,800	0	0	0.225	0.159
" 23	35	75	85	1,201				920	0	0	Positive.	
" 24	35	75	85	1,205	46.6	31		1,640	0	0	"	
" 25	35	75	85	1,205				1,130	0	0	Negative.	
" 26	35	75	85	1,205				1,020	0	0	0.322	0.238
" 27	35	75	85	1,205	45.6							

Under "acetone" the results of determinations of acetone plus aceto-acetic acid are listed.

Results of the determinations of the acetone bodies are expressed in terms of acetone.

Results of tests with sodium nitroprusside and ammonia are given for specimens on which quantitative determinations of the acetone bodies were not made.

stant on an intake of food which furnished a smaller number of calories
than would have formed the basal requirement of a normal subject of the
same height and weight. The acetone excretion found in this case was
fairly constant throughout the study, and was rather lower than was to
be expected from the value of the ratios—60 per cent—calculated from the
diet. It seems probable from a study of weight changes that in this case
120 per cent of the calculated basal requirement represents more calories
than the patient actually burned in a day. He was the only patient in
the series who was confined to his bed, and the only patient who main-
tained his body weight on such a low food intake. If the calculation of
the ratio glucose to fat burned had been based on the diet which main-
tained weight instead of arbitrarily on 120 per cent of his calculated basal
requirement, the value of the ratio which corresponded to an excretion
of 0.5 gm. of acetone would have been 70 to 75 per cent. There was no
reason to believe that this patient did not follow the diet, but such a possi-
bility cannot be absolutely excluded.
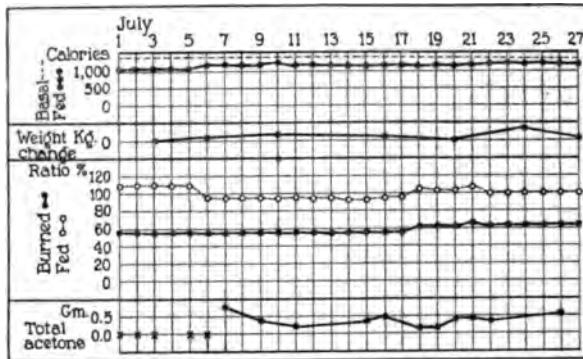
CHART 5. Case 5.

Case 6 was a patient who had had diabetes for 14 years, and when he
was studied his condition was rather severe. The patient received less
food than would furnish calories equivalent to the basal requirement of
a normal subject of the same height and weight, and showed a progressive
loss of weight during the period. Increased amounts of acetone were
found in almost all the specimens of urine analyzed, but the amounts were
small, as compared with those expected from the numerical values of the
ratio. Two analyses of the nitrogen content of the urine on the 7th and
the 27th of July by the method of Folin and Denis (1916) (slightly modified
to permit the use of the oxidizing and Nessler's reagent described by
Folin and Wu (1919)) showed that the patient was approximately in
nitrogen equilibrium on these dates. The subject was not confined to his
bed, and there is no reason for believing that his caloric requirements
during the day were less than those of the first four cases discussed. No

TABLE VI.

Case 6.

Date	Diet.				Weight.	Alveolar CO₂.	Blood sugar.	Urine.				β-hydroxybu-tyric acid.
	Carbohydrate.	Protein.	Fat.	Calories.				Volume.	Sugar.		Acetone.	
1921	gm.	gm.	gm.		kg.	mm.	per cent	cc.	per cent	gm.	gm.	gm.
June 23	?	?	?	?				?	3.0		Positive.	
" 24	102	58	0	640	57.0		0.330	1,200	2.3	27.6	"	
" 25	50	75	70	1,130				1,740	0.7	12.0	"	
" 26	25	38	35	567	55.8			1,750	0.2	4.4	Negative.	
" 27	33	50	47	755				1,380	Trace.	3.4	Positive.	
" 28	33	50	47	755				1,580	"		"	
" 29	33	50	47	755	55.6			1,830	0	0	"	
" 30	26	69	50	830				1,820	0	0	Negative.	
July 1	25	70	48	812				1,750	0	0	Positive.	
" 2	30	70	60	940			0.225	1,860	0	0	"	
" 3	30	70	70	1,030	54.7			1,260	0	0	"	
" 4	30	70	70	1,030				1,240	0	0	"	
" 5	35	70	75	1,095				1,540	0	0	Negative.	
" 6	35	71	80	1,144	55.4			1,140	0	0	"	
" 7	35	70	85	1,185				1,000	0	0	0.238	0.187
" 8	37	70	85	1,185			0.230	950	0	0	Negative.	
" 9	18	36	43	603		30		910	0	0	0.090	0.106
" 10	31	40	38	626	54.3			1,240	0	0	Positive.	
" 11	20	44	50	706				1,480	0	0	0.075	0.148
" 12	20	46	50	714			0.200	1,450	0	0	Positive.	
" 13	21	55	55	799	54.3			1,500	0	0	0.382	0.890
" 14	20	60	55	810				945	0	0	Positive.	
" 15	25	59	54	822		30		1,200	0	0	0.221	0.342
" 16	30	66	54	870				1,620	0	0	0.238	0.264
" 17	34	69	60	952		30		1,560	0	0	Positive.	
" 18	35	69	60	956				1,270	0	0	0.159	0.306
" 19	35	74	60	1,020			0.200	1,300	0	0	0.130	0.076
" 20	35	75	74	1,106	53.3			1,700	0	0	0.137	0.102
" 21	35	80	79	1,171				1,330	0	0	0.114	0.082
" 22	35	80	80	1,180		33		1,310	0	0	0.130	0.127
" 23	35	80	80	1,180				1,400	0	0	Positive.	
" 24	35	80	80	1,180	53.3	34		1,140	0	0	"	
" 25	35	79	80	1,176				1,330	0	0	"	
" 26	35	79	80	1,176				1,330	0	0	0.124	0.162
" 27	35	80	80	1,180	52.4		0.200	960	0	0	0.077	0.117

Under "acetone" the results of determinations of acetone plus aceto-acetic acid are listed.

Results of the determinations of the acetone bodies are expressed in terms of acetone

Results of tests with sodium nitroprusside and ammonia are given for specimens on which quantitative determinations of acetone were not made.

adequate explanation can be offered for the low acetone excretion found in studying this patient.
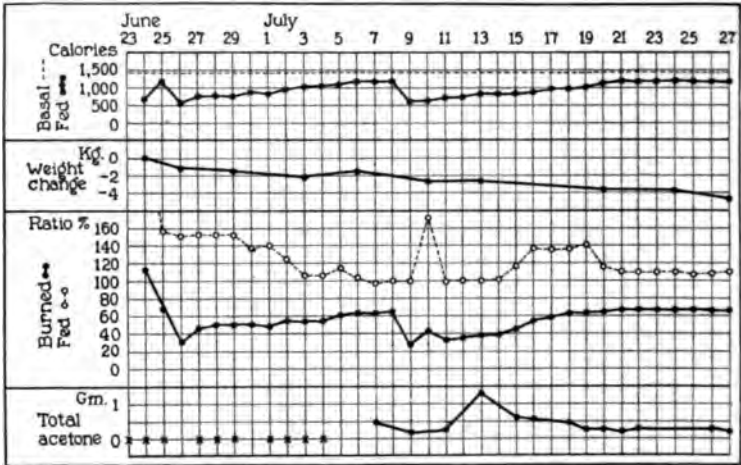
CHART 6. Case 6.
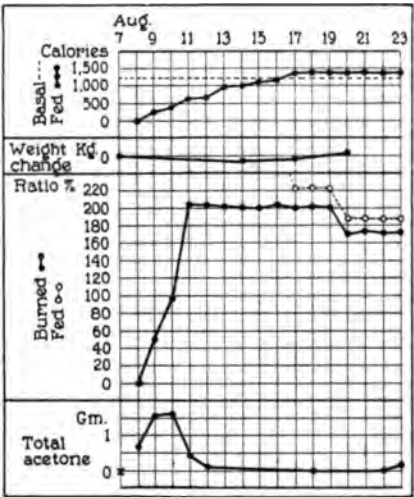
CHART 7. Case 7.

Case 7 had a very mild case of diabetes of short duration. The food provided furnished 115 per cent more calories than her calculated requirement, and her weight was constant, but the period of observation was too

short to prove this requirement sufficient. The most striking feature shown by this case is a slight return of acetonuria—demonstrated both by quantitative and qualitative tests—when the ratio of glucose to fat burned passed from a value of 200 per cent to one of 170 per cent. This appearance of increased amounts of acetone when the ratio has such a high

TABLE VII.

Case 7.

Date.	Diet.				Weight.	Alveolar CO₂.	Blood sugar.	Urine.			
	Carbohydrate.	Protein.	Fat.	Calories.				Volume.	Sugar.	Acetone.	β-hydroxybutyric acid.
1921	gm.	gm.	gm.		kg.	mm.	per cent	cc.	per cent	gm.	gm.
Aug. 7	?	?	?	?	44.6	37		?	6.6	?	Positive.	
" 8	0	0	0	0		31		2,300	0	0	0.211	0.498
" 9	45	24	0	266		29	0.140	2,730	0	0	0.506	1.015
" 10	64	33	0	388				1,970	0	0	0.754	0.812
" 11	102	58	0	640				1,800	0	0	0.165	0.247
" 12	102	58	0	640		34		2,050	0	0	0.051	0.059
" 13	100	60	30	910				1,820	0	0	Negative.	
" 14	100	60	40	1,000	44.0			1,820	0	0	"	
" 15	100	60	50	1,090		35		1,940	0	0	"	
" 16	101	60	60	1,184				2,300	0	0	"	
" 17	100	60	80	1,360	44.3			1,980	0	0	"	
" 18	100	61	80	1,364			0.175	1,300	0	0	0.019	0.018
" 19	100	60	80	1,360		36		1,500	0	0	Negative.	
" 20	80	60	90	1,374				1,440	0	0	"	
" 21	80	61	90	1,374	45.0			1,730	0	0	Positive.	
" 22	80	61	90	1,374				1,420	0	0	0.021	0.025
" 23	80	61	90	1,374				1,430	0	0	0.108	0.081

Under "acetone" the results of the determinations of acetone plus acetoacetic acid are listed.

Results of the determinations of the acetone bodies are expressed in terms of acetone.

Results of the test with sodium nitroprusside and ammonia are given for specimens on which quantitative analyses for the acetone bodies were not made.

value forms a marked contrast to the results obtained in the case just described, in which only small amounts of acetone were found when the ratio had a value of 60 per cent. The results in this case are similar to those found in studying one of the normal subjects as reported in a previous paper (Hubbard and Wright, 1922).

DISCUSSION.

A comparison of the acetone excretion with the molecular ratio used to express the ketogenic balance of the diets ingested shows that, in general, the excretion varied inversely with the value of the ratio based on the probable amount of fat burned by the patient during 24 hours. The amounts of acetone found were somewhat smaller than those which were found in the study of normal patients who were receiving diets which gave ratios of the same numerical values, but the border-line diet which caused a very slight increase in acetone excretion lay approximately at a value of 80 per cent, as it did in the earlier experiments. Traces of acetone were found when diets having a higher ratio were fed— as traces were occasionally found under similar conditions in some of the experiments run on normal subjects; in one of the patients studied such traces were found when the ratio had a value of 170 per cent. It seems reasonable to attribute such findings, as was done in the earlier paper, to temporary excess of ketogenic material which may have lasted for only a comparatively small part of the 24 hour period, or to a local excess of such material due to variations in the blood and nutriment supplied to different parts of the organism. One patient showed an excretion of acetone which was lower than was expected from theoretical considerations or from a comparison with other cases in the series; the tolerance of this patient for glucose was so low that it was not possible to investigate the diet which would cause no excretion of acetone.

The excretion of acetone could be largely explained by a study of the molecular ratio based on the amount of fat probably burned by the patient, but increases were sometimes found which could not be accounted for in this way. An increase in fat fed was followed by an increased excretion of acetone in some cases when this was the only change in the diet, and the additional fat fed theoretically replaced fat which the subject had been drawing from his own reserves. Such a change in the diet was shown in the numerical method used for expressing the diets, by a decrease in the value of the ratio based upon the amount of fat fed while the ratio based upon the probable amount of fat burned was not changed; in these instances the fed fat replaced only a part of the total fat

burned—a condition in which the values of the ratio based upon the amount of total fat burned are lower than those based upon the amount of fat fed. The increases in acetone excretion under these conditions were not large, and did not seem to last long. If the organism had not been at first able to burn completely the large amounts of fat received at intervals with the meals, but had later acquired the ability to do so, results similar to those found would have been expected, and some such explanation may account for the temporary increases in acetone excretion noted. This appearance of increased amounts of acetone with increased intake of fat, even when the fat probably only served to replace fat withdrawn from the body reserves, made it seem inadvisable to furnish more fat than that which corresponds with Woodyatt's (1921) formula for the border-line diet

$$2 \times \text{carbohydrate} + \tfrac{1}{2} \text{protein} = \text{fat}$$

except in cases in which such increases were necessary to maintain life.

CONCLUSIONS.

A method has been described for calculating a molecular ratio between ketogenic and antiketogenic compounds contained in the diet which is applicable to diabetic patients, and seven cases have been described in which a comparison was made between the values of this ratio and the excretion of the acetone bodies in the urine; it has been shown that the acetone excretion varies inversely with the numerical values of this ratio; a study of the numerical values of the ratio calculated for diets which correspond with a slightly increased excretion of acetone shows that they were approximately the same as those values found for normal subjects receiving diets low in carbohydrate, but containing sufficient calories to supply the needs of the subject; it has been shown that fat fed sometimes increases the amount of acetone excreted, even when the increase replaces a part of the fat which the body was probably withdrawing from its own reserve supplies of this material.

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THE PROTEINS OF THE LIMA BEAN, PHASEOLUS LUNATUS.

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In continuation of the comparative studies of the proteins of various beans, particularly those of the genus *Phaseolus*, a study of the proteins of the lima bean is described in this paper. As far as the authors are aware, the only previous reference to any work done on this bean is a statement by Osborne (1) to the effect that some preliminary analyses which he made indicated that the chief protein constituent of this bean is the same as phaseolin obtained from the navy bean.

The results of the work described in the present study show that the proteins of the lima bean are in general quite similar to those of the other beans which have been studied: namely, the navy, *Phaseolus vulgaris*;[1] mung, *Phaseolus aureus* (2); Chinese velvet, *Stizolobium niveum* (3); Georgia velvet, *Stizolobium deeringianum* (4); aduski, *Phaseolus angularis* (5); and the jack bean, *Canavalia ensiformis* (6). Like these, the lima bean contains two globulins which differ characteristically in both sulfur and nitrogen contents. Feeding experiments with albino rats have also shown (7) a similarity in their nutritive properties. The lima bean is also deficient in cystine, and its proteins are characterized by a form of indigestibility which is remedied by cooking. On the other hand, the proteins of the lima bean differ in certain respects from those of the other beans studied, the most striking difference being the low nitrogen content of the β-globulin. The average of closely agreeing results for seven different preparations of this protein showed it to contain 14.81 per cent of nitrogen (Table III). The

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[1] Studies on the proteins of the navy bean are nearly completed and the results will be published later.

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proteins of this bean are also marked by a rather high degree of solubility in saline solutions. The soluble salts occurring naturally in the seeds are evidently sufficient to dissolve 15.13 per cent of protein when the meal is extracted with distilled water in the proportion of 2.5 cc. per gram of meal. This is practically as much as was extracted by a 3 per cent sodium chloride solution (Table I).

The meal used for the preparation of the proteins described in this paper was obtained from beans of two varieties, the "Fordhook bush" and "Carpenteria pole," which were obtained in the open market. No differences were observed in the results obtained from these two varieties. The meal contained 21.17 per cent of protein (N × 6.25). 3 per cent sodium chloride solution at room temperature extracted 72.32 per cent of the total protein in the meal, or 15.31 per cent based on the amount of meal used.

The α- and β-globulins were separated by fractional precipitation from 3 per cent sodium chloride solution by means of ammonium sulfate. The α-globulin was precipitated by addition of ammonium sulfate until the solution was brought up to 0.25 of saturation (19.07 gm. of ammonium sulfate per 100 cc. of the final solution). The β-globulin separated between 0.45 and 0.75 of saturation.

An albumin, amounting to 1.75 per cent of the meal, or 8.26 per cent of the total protein, was obtained by boiling a distilled water extract of the meal after all of the globulins had been removed. This percentage of albumin is considerably higher than that obtained from the other beans which have been studied.

The percentages of the basic amino-acids in the three proteins isolated were determined by Van Slyke's method, with results as given in Tables VI, VIII, and X.

<center>EXPERIMENTAL.</center>

Preliminary Experiments.—Extraction experiments were made with the bean meal, using various concentrations of sodium chloride in water in the proportion of 2.5 cc. of solvent to each gram of meal. The mixtures were allowed to stand for 3 hours at room temperature, with shaking at intervals of about 15 minutes. Nitrogen determinations made on the filtered extracts showed that the maximum amount of protein was extracted by a 3 per cent solution of sodium chloride (Table I).

Preparation of the α-Globulin.—For each preparation of the α-globulin about 3.5 kilos of meal were extracted with 3 per cent sodium chloride solution. Filter paper scraps were then pulped in the mixtures until a consistency suitable for pressing was obtained, and the mixtures were pressed in muslin bags. The expressed liquors were filtered clear by suction through a mat of paper pulp, and the filtrates made 0.25 saturated by addition of solid ammonium sulfate. After standing over night the precipitates were collected on folded filter papers and washed with 3 per cent sodium chloride solution which had been previously made 0.25 saturated with ammonium sulfate. Since these precipitates required very large volumes of the sodium chloride solution to redissolve them, they were dialyzed in suspension in this solvent against chilled running water for 11 days. The proteins which had separated were washed with distilled water until free from chlorides and sulfates, and dried with alcohol and ether in

TABLE I.

*Extraction Experiments.**

Solvent.	Protein extract from meal (N × 6 25).
	per cent
Distilled water...	15.13
1.0 per cent NaCl......................................	15.19
3.0 " " "	15.31
8.0 " " "	14.50
10.0 " " "	14.06
15.0 " " "	13.63
20.0 " · " "	11.84

* The extractions were carried out at room temperature, for a period of 3 hours each, with frequent stirring. Solvents were used in the proportion of 2.5 cc. per gm. of meal.

the usual way. An average yield of 2.74 per cent of the total protein, or 0.58·per cent of the meal used, was obtained. Average results of duplicate analyses of five preparations of the α-globulin are given in Table II. The analyses are calculated on an ash- and moisture-free basis.

Preparation of the β-Globulin.—A small intermediate fraction consisting of a mixture of the α- and β-fractions was removed and discarded. This fraction was obtained by bringing the filtrates from the original precipitates of the α-globulin, which were already 0.25 saturated, up to 0.4 of saturation with ammonium sulfate. The filtrates from the intermediate fraction were then made 0.75 saturated by addition of more ammonium sulfate, and the precipitated β-globulin was filtered and washed in the manner described in the case of the α-globulin. The precipitates were then redissolved in the minimum amount of distilled water and dialyzed for 13 days. An average yield of 1.58 per cent of the meal extracted was obtained.

Seven preparations were made which gave closely agreeing results on elementary analyses. Average results of duplicate analyses are given in Table III.

TABLE II.

*Average Results of Duplicate Analyses of the α-Globulin.**

| | Preparation. | | | | | |
	I	II	III	IV	V	Average.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
C..............	53.42	53.66	53.69	53.63	53.87	53.65
H..............	6.88	6.60	6.59	6.74	6.45	6.65
N..............	15.84	15.57	15.56	15.28	15.50	15.55
S..............	1.29	1.23	1.27	1.28	1.30	1.27
O..............	22.57	22.94	22.89	23.07	22.88	22.88
Moisture.......	5.44	7.52	7.25	7.36	7.77	
Ash...........	1.52	0.44	0.65	0.43	0.57	

* Calculated on an ash- and moisture-free basis.

TABLE III.

*Average Results of Duplicate Analyses of the β-Globulin.**

| | Preparation. | | | | | | | |
	I	II	III	IV	V	VI	VII	Average.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
C..................	52.59	52.61	52.80	52.70	52.78	52.72	52.85	52.72
H..................	6.61	6.78	6.83	6.83	6.74	6.74	6.83	6.77
N..................	14.89	14.90	14.85	14.60	14.82	14.80	14.85	14.81
S..................	0.37	0.33	0.35	0.36	0.37	0.34	0.36	0.35
O..................	25.54	25.38	25.17	25.51	25.29	25.40	25.11	25.35
Moisture...........	8.76	6.96	9.27	5.66	6.58	7.99	6.76	
Ash................	0.80	1.08	0.61	0.49	0.44	0.71	0.69	

* Calculated on an ash- and moisture-free basis.

Properties of the Globulins.—Both globulins, when prepared and dried as described, consisted of dusty powders. The α-globulin had a deep cream color, while the β-globulin was pure white. The two globulins are fairly well differentiated by their precipitation limits with ammonium sulfate. Precipitation of the α-globulin began at 0.15 of saturation and became flocculent at 0.25 of

saturation.   Between 0.3 and 0.4 of saturation a small precipitate was obtained, which had a sulfur content of 0.63 per cent, indicating that it was a mixture of the two globulins.   The β-globulin precipitated at 0.45 to 0.75 of saturation.

Coagulation temperatures of the globulins, determined on their saline extracts which had been slightly acidified with dilute acetic acid, showed that the α-globulin coagulated at about 68° C. and the β-globulin at about 95° C.

Both globulins gave positive tests for tryptophane with the Hopkins and Cole reagent (8), the color developing immediately in the case of the β-globulin, while the α-globulin required more time for the color development.

TABLE IV.

*Average Results of Duplicate Analyses of the Albumin.**

	Preparation.		
	I	II	Average.
	per cent	*per cent*	*per cent*
C.........................	54.14	54.19	54.17
H.........................	6.62	6.64	6.63
N.........................	14.20	14.24	14.22
S.........................	1.15	1.15	1.15
O.........................	23.89	23.78	23.83
Moisture.................	9.21	8.72	
Ash.....................	1.68	3.19	

* Calculated on an ash- and moisture-free basis.

The Albumin.—2 liters of water and 500 gm. of meal were mixed and passed three times through a peanut grinder. The mixture was then pressed, filtered in the usual way, and the clear extract dialyzed for 5 days. The precipitated globulins were removed by filtration, and the filtrate was again dialyzed for 8 days. After removing a small amount of precipitated globulin, the solution was saturated with carbon dioxide which caused the further separation of a small amount of precipitate. This substance was filtered and the albumin was coagulated by boiling the filtrate which had been previously slightly acidified with acetic acid. After washing with hot water and drying in the usual way at 55°C., the albumin consisted of a cream white amorphous powder. The yield amounted to 1.75 per cent of the meal. The average results of duplicate analyses of two preparations are given in Table IV. The albumin gave a faint, though decided, test for tryptophane.

Analyses of the α- and β-Globulins and of the Albumin by the Van Slyke Method.—Duplicate samples of 3 gm. each of the globulins and albumin were hydrolyzed by boiling for about 30 hours with

TABLE V.

*Distribution of Nitrogen in the α-Globulin as Determined by the Van Slyke Method.**

Sample I, ash- and moisture-free, 2.7630 gm. protein, 0.4299 gm. nitrogen.†
   "   II,   "   "   "   "   2.7630   "   "   0.4299   "   "

	Preparation.				
	I	II	I	II	Average.
	gm.	*gm.*	*per cent*	*per cent*	*per cent*
Amide N	0.0442	0.0444	10.28	10.33	10.31
Humin N adsorbed by lime	0.0086	0.0087	2.00	2.02	2.01
Humin N in amyl alcohol-ether extract	0.0008	0.0010	0.19	0.23	0.21
Cystine N	0.0052	0.0051	1.21	1.19	1.20
Arginine N	0.0506	0.0502	11.77	11.68	11.72
Histidine N	0.0276	0.0279	6.42	6.49	6.46
Lysine N	0.0418	0.0413	9.72	9.61	9.67
Amino N of filtrate	0.2389	0.2400	55.57	55.82	55.69
Non-amino N of filtrate	0.0127	0.0122	2.95	2.84	2.89
Total N regained	0.4304	0.4308	100.11	100.21	100.16

* Nitrogen figures corrected for the solubility of the bases.
† Nitrogen content of protein, 15.56 per cent.

TABLE VI.

Basic Amino-Acids in the α-Globulin.

Amino-acid.	I	II	Average.
	per cent	*per cent*	*per cent*
Cystine	1.61	1.58	1.60
Arginine	5.69	5.65	5.67
Histidine	3.69	3.73	3.71
Lysine	7.89	7.80	7.84
Tryptophane			Present.

100 cc. of 20 per cent hydrochloric acid. The phosphotungstates of the bases were decomposed by the amyl alcohol-ether method (9). The results of the analyses are given in Tables V to XI.

TABLE VII.

*Distribution of Nitrogen in the β-Globulin as Determined by the Van Slyke Method.**

Sample I, ash- and moisture-free, 2.7390 gm. protein, 0.4054 gm. nitrogen.†
"   II, "   "   "   "   2.7390 "   "   0.4054 "   "

	Preparation.				
	I	II	I	II	Average.
	gm.	*gm.*	*per cent*	*per cent*	*per cent*
Amide N...............................	0.0414	0.0412	10.21	10.17	10.19
Humin N adsorbed by lime.............	0.0062	0.0065	1.54	1.60	1.57
Humin N in amyl alcohol-ether extract ..	0.0003	0.0003	0.07	0.07	0.07
Cystine N.............................	0.0027	0.0027	0.67	0.67	0.67
Arginine N............................	0.0443	0.0450	10.93	11.10	11.02
Histidine N...........................	0.0199	0.0190	4.91	4.69	4.80
Lysine N..............................	0.0450	0.0445	11.10	10.98	11.04
Amino N of filtrate....................	0.2422	0.2422	59.75	59.75	59.75
Non-amino N of filtrate...............	0.0060	0.0066	1.48	1.63	1.55
Total N regained.....................	0.4080	0.4080	100.66	100.66	100.66

* Nitrogen figures corrected for the solubility of the bases.
† Nitrogen content of protein, 14.80 per cent.

TABLE VIII.

Basic Amino-Acids in the β-Globulin.

Amino-acid.	I	II	Average.
	per cent	*per cent*	*per cent*
Cystine..................	0.84	0.84	0.84
Arginine.................	5.03	5.11	5.07
Histidine................	2.68	2.56	2.62
Lysine..................	8.57	8.48	8.53
Tryptophane.............			Present.

TABLE IX.

*Distribution of Nitrogen in the Albumin as Determined by the Van Slyke Method.**

Sample I, ash- and moisture-free, 2.6427 gm. protein, 0.3763 gm. nitrogen.†
"   II,   "   "   "   "   2.6427 "   "   0.3763 "   "

	Preparation.				
	I	II	I	II	Average.
	gm.	gm.	per cent	per cent	per cent
Amide N................................	0.0367	0.0369	9.75	9.81	9.78
Humin N adsorbed by lime.............	0.0070	0.0070	1.86	1.86	1.86
Humin N in amyl alcohol-ether extract..	0.0007	0.0007	0.19	0.19	0.19
Cystine N.............................	0.0033	0.0033	0.88	0.88	0.88
Arginine N............................	0.0488	0.0488	12.97	12.97	12.97
Histidine N............................	0.0188	0.0179	5.00	4.76	4.88
Lysine N...............................	0.0298	0.0307	7.92	8.16	8.04
Amino N of filtrate.....................	0.2358	0.2358	62.66	62.66	62.66
Non-amino N of filtrate................	0.0023	0.0023	0.61	0.61	0.61
Total N regained.....................	0.3832	0.3834	101.84	101.90	101.87

* Nitrogen figures corrected for the solubility of the bases.
† Nitrogen content of protein, 14.24 per cent.

TABLE X.

Basic Amino-Acids in the Albumin.

Amino-acid.	I	II	Average.
	per cent	per cent	per cent
Cystine..................	1.07	1.07	1.07
Arginine.................	5.74	5.74	5.74
Histidine................	2.63	2.44	2.54
Lysine..................	5.88	6.06	5.97
Tryptophane............			Present.

TABLE XI.

*Distribution of Nitrogen in the α- and β-Globulins, and in the Albumin as
Calculated from the Van Slyke Analyses in Terms of Percentage
of the Proteins.*

N	α-Globulin.*			β-Globulin.†			Albumin.‡		
	I	II	Average.	I	II	Average.	I	II	Average.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Amide..................	1.60	1.61	1.61	1.51	1.50	1.51	1.39	1.40	1.40
Humin................	0.34	0.35	0.34	0.24	0.25	0.24	0.29	0.29	0.29
Basic..................	4.53	4.51	4.52	4.09	4.06	4.08	3.81	3.81	3.81
Non-basic............	9.11	9.13	9.12	9.06	9.08	9.07	9.01	9.01	9.01
Total...............	15.58	15.60	15.59	14.90	14.89	14.90	14.50	14.51	14.51

* Nitrogen content, 15.56 per cent.
† Nitrogen content, 14.80 per cent.
‡ Nitrogen content, 14.24 per cent.

SUMMARY.

The lima bean meal used for the extraction of the proteins contained 21.17 per cent of protein (N × 6.25). 3 per cent sodium chloride solution at room temperature extracted 72.32 per cent of the total protein, or 15.31 per cent based on the weight of the meal used.

Two globulins were isolated by fractional precipitation of the sodium chloride extracts by means of ammonium sulfate. The α-globulin was precipitated by addition of ammonium sulfate until the original extract was 0.25 saturated. The β-globulin separated between 0.45 and 0.75 of saturation. A small fraction intermediate between the α- and β-globulins was removed and discarded. This fraction consisted of a mixture of the two globulins.

An albumin, amounting to 1.75 per cent of the meal, or 8.25 per cent of the total protein, was obtained from distilled water extracts of the bean meal after the globulins had been removed.

Elementary analyses of the three proteins isolated and determination of the basic amino-acids by the Van Slyke method show in general the same differences as have been found between the corresponding proteins obtained from other beans which have been

studied.   Both globulins and the albumin gave positive tests for tryptophane.

As shown in a previous publication on the nutritive value of the proteins of the lima bean, its total proteins are deficient in cystine and are characterized by a form of indigestibility which is remedied by cooking.

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THE DISTRIBUTION OF SODIUM, POTASSIUM, CALCIUM, AND MAGNESIUM BETWEEN THE CORPUSCLES AND SERUM OF HUMAN BLOOD.

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A knowledge of the concentration of sodium, potassium, calcium, and magnesium in the blood is necessary in connection with a variety of problems. Methods hitherto used for the quantitative determination of these elements in blood have required large amounts of material even for a single determination and have been, in most cases, difficult to carry out. These facts have tended to discourage such studies, particularly with human blood.

The earliest recorded figures for the concentration of cations and anions in human blood, corpuscles, and serum, are those reported by Schmidt (1) in 1850. Wanach (2) in 1888 estimated the sodium and potassium in the blood corpuscles and serum of eight adults. Some years before this Bunge (3), a pupil of Schmidt, reported a number of complete analyses of the ash of the blood corpuscles and serum of several animals. Abderhalden (4) later published the results of a similar study on a larger series of animals. No studies on the distribution of cations between the corpuscles and serum of normal human blood have appeared since those of Wanach.[1]

The introduction of methods for the quantitative determination of sodium, potassium, calcium, and magnesium with small amounts of serum and whole blood (5 to 9) has made such studies on human

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[1] After this paper had been written an article appeared on the effect of changes in CO_2 tension upon the distribution of sodium, potassium, chlorine, phosphorus, and bicarbonate between the corpuscles and plasma of defibrinated and filtered beef blood (Doisy, E. A., and Eaton, E. P., *J. Biol. Chem.*, 1921, xlvii, 377).

subjects possible. We have investigated the concentration of these elements in the venous blood and serum of normal adults. The determinations were made on serum in preference to plasma because the addition of an anticoagulant (usually a salt) is thereby avoided. This permits the determination of all the cations to be made on the same sample. Hemolysis occurs less frequently with serum than plasma. We have found the potassium and calcium content of citrated plasma to be practically the same as that of serum. Schmidt (1) has shown that the inorganic composition of plasma is identical with that of serum.

We have repeatedly demonstrated the remarkable constancy of the concentration of the elements (sodium, potassium, calcium,

TABLE I.

Concentration of Sodium in Blood Serum and Corpuscles of Normal Adults.

Sample.	Plasma.	Na per 100 cc. serum.	Na per 100 cc. blood.	Na in plasma of 100 cc. of blood.	Na in 100 cc. corpuscles calculated.
	per cent	*mg.*	*mg.*	*mg.*	*mg.*
1	59	335	193	198	−12
2	65	335	220	218	+ 6
3	58	335	195	194	+ 3
4	57	335	186	191	−12
5	58	335	187	194	−17
6	56	335	189	187	+ 5
7	60	335	199	201	− 5

and magnesium), in the serum of normal adults and have therefore assumed in Tables I and II the average of our previously reported figures for sodium and potassium as representing the concentration of these elements in the serum of the normal adult male. The concentration of calcium was determined directly on ashed plasma. The whole blood samples were collected in distilled water and weighed and the cations determined by methods referred to above. The relative proportion of corpuscles to plasma was determined by the use of hematocrit, and the concentration of the various elements in the corpuscles calculated from the assumed concentration of the respective element in the serum, its concentration in whole blood, and the percentage of corpuscles.

The results of our determinations are given in Tables I to VI.

Column 2 of Table I shows the proportion of plasma in the blood samples analyzed; Column 3, the average value for sodium expressed in mg. per 100 cc. of serum for all our determinations with sera of normal adults. The figures for sodium of whole blood are given in Column 4. The number of mg. of sodium present in the amount of serum contained in 100 cc. of the given whole blood sample, is given in Column 5. It will be seen that the latter is practically the same as the sodium concentration in the whole blood of the sample; *i.e.*, that there is no sodium in the corpuscles.

TABLE II.

Concentration of Potassium in Blood Serum and Corpuscles of Normal Adults.

Sample.	Plasma.	K per 100 cc. serum.	K per 100 cc. blood.	K per 100 cc. corpuscles.
	per cent	*mg.*	*mg.*	*mg.*
1	61	19.5	172	410
2	60	19.5	187	438
3	57	19.5	188	410
4	68	19.5	153	437
5	58	19.5	186	420
6	57	19.5	200	438
7	61	19.5	180	430
8	62	19.5	175	428
9	57	19.5	202	444
10	59	19.5	193	441
11	65	19.5	164	413
12	65	19.5	169	425
13	56	19.5	201	430
Average.....	60.5	19.5	182	428

Column 4 of Table II gives the figures for the number of mg. of potassium per 100 cc. of whole blood. We have calculated the concentration of the same element in 100 cc. of corpuscles and placed the results in Column 5. It is evident that the K content of the blood of the adult man varies directly as the corpuscular content. With a corpuscular content of 43 per cent (Sample 3) the K of 100 cc. of whole blood was 188 mg. whereas when the percentage of corpuscles dropped to 32 (Sample 4) the K content of the whole blood fell to 153 mg. per 100 cc. This is shown in a more striking manner if one calculates the K content of the corpuscles. When this is done the constancy of the K content of the

corpuscles becomes apparent (see Column 5).   Since, as we shall
see later, the corpuscles contain no calcium and only traces of
magnesium and, as we have seen from results recorded in Table I,
no sodium, it becomes obvious that potassium constitutes practi-
cally all the fixed mineral base of the corpuscles.   In this respect
man differs from a number of animals, namely the dog and cat,

TABLE III.

Concentration of Calcium in Blood Serum and Corpuscles of Normal Adults.

Sample.	Plasma.	Ca per 100 cc. plasma.	Ca per 100 cc. blood.	Calcium in plasma of 100 cc. blood.	Ca in 100 cc. of corpuscles calculated.
	per cent	*mg.*	*mg.*	*mg.*	*mg.*
1	58	10.0	5.3	5.8	−1.2
2	57	9.5	5.3	5.4	−0.2
3	72	9.5	6.7	6.8	−0.4
4	59	9.8	6.2	5.8	+1.0
5	58	9.5	5.3	5.4	−0.2
6	65	9.3	5.9	6.0	−0.3
7	57	9.7	5.5	5.5	±0.0

TABLE IV.

Concentration of Magnesium in Blood and Serum of Normal Adults.

Sample.	Magnesium per 100 cc. whole blood.	Magnesium per 100 cc. serum.
	mg.	
1	2.6	
2	4.0	
3	3.8	
4	3.8	
5	2.8	
6	2.8	
7	3.8	
8	2.3	
Average.............	3.2	2.5

whose corpuscles contain potassium in practically the same con-
centration as does their plasma.   These animals make up their
deficit of potassium in the corpuscles with sodium.

Table III shows that in seven consecutive instances, we analyzed
the calcium of the serum and whole blood and calculated from
these data and the hematocrit reading the calcium of the cor-

puscles. In not a single instance were we able to demonstrate, with certainty, the presence of calcium in the corpuscles.

Table IV requires no explanation.

SUMMARY.

1. We have previously shown that the concentrations of sodium, potassium, calcium, and magnesium in the sera of normal adults and children are singularly constant.

2. Table I shows that human corpuscles are practically free of sodium.

3. The concentration of potassium in human corpuscles is remarkably constant varying only from 410 to 440 mg. per 100 cc. of corpuscles. The average value found for thirteen samples was 428 mg. This is about twenty times the concentration of the same element in serum. Potassium represents practically all the fixed mineral base of human corpuscles.

4. Only about 2 to 4 mg. of magnesium are present in 100 cc. of corpuscles.

5. The magnesium content of whole blood varies from 2.3 to 4.0 mg. per 100 cc. These figures agree with those reported by earlier investigators.

6. In a study of seven consecutive normal bloods we found practically no calcium in the corpuscles.

The Presence of Calcium in Corpuscles.—The question of the presence of calcium in blood corpuscles has recently been the subject of considerable discussion. The earlier workers (4) regularly found no calcium in the corpuscles. More recently a number of investigators have reported the finding of considerable amounts of calcium in blood corpuscles. Hamburger (10) found as much as 32 mg. of calcium per 100 cc. of corpuscles. Rona and Takahashi (11) were able to demonstrate the presence of only 1.0 to 2.4 mg. in an equal volume of corpuscles while Heubner and Rona (12) found demonstrable amounts of calcium in the red blood cells of only six cats in a series of twenty-six animals studied. Cowie and Calhoun (13) maintain that considerable amounts of calcium are present in corpuscles and in more recent publications Jones and Nye, and Jones (14) insist upon the presence of calcium in corpuscles in appreciable amounts.

Howland and Marriott (15) found human corpuscles to be free of calcium.   Lamers (16) concluded from his analyses of the blood of healthy women and of women suffering with a variety of diseases, that detectable amounts of calcium do not occur in human corpuscles.   Richter-Quittner and Falta (17) also failed to find calcium either in human blood corpuscles or in those of animals.   In a series of seven consecutive samples of blood from seven normal adults we have likewise been unable to demonstrate the presence of calcium in the corpuscles of a single individual. We shall not enter here into a description of the various controls which we have made to convince ourselves of the accuracy of our methods both for serum as well as for whole blood.   These have been described elsewhere in detail.   We are convinced, and our conviction is based upon a large number of analyses of human blood, and of the blood of a variety of animals, that the calcium of serum or plasma is remarkably constant for a large variety of normal animals (man, dog, rat, sheep, and cow), varying only from 9 to 11 mg. per 100 cc. of serum.   The concentration of the same element in whole blood shows a much greater fluctuation because of the variation in the percentage of corpuscles in different samples.   Nevertheless, the figure for whole blood rarely exceeds 7 mg. per 100 cc. of blood.   Neither does it fall, except in cases of marked polycythemia, below 5 mg.   Table III illustrates some of these statements.   We have been unable to demonstrate the presence of calcium in appreciable amounts in the corpuscles of the normal adult and are inclined to attribute the finding of calcium in the corpuscles by others to errors in their calcium determinations.   The sources of such errors in the determination of minute amounts of calcium have been discussed by Kramer, Tisdall, and Howland (18).

A simple example in which the figures used represent the average of a large series of determinations made by Jones and Nye (14) on the blood of normal boys and girls, will serve to illustrate the usual error in such investigations.   The average calcium concentration per 100 cc. of plasma was found by them to be 10.1 mg., that of whole blood 9.4 mg., and the percentage of corpuscles. was 38.4.   If there were no calcium in the corpuscles the concentration of calcium per 100 cc. of whole blood should have been 10.1 × 61.6 = 6.2 mg.   A glance at Table III shows this to be a

normal value for the calcium concentration of whole blood. The amount actually found by Jones and Nye was 9.4 mg. They used the method of Lyman (19) for their calcium determinations. It has been pointed out elsewhere (18) that when the supernatant fluid obtained after precipitating plasma proteins with trichloro-acetic acid (as performed in the Lyman procedure) is filtered through even good grades of acid-washed filter paper, calcium, in variable, but demonstrable amounts, may enter the filtrate and be responsible for many of the high values for calcium frequently obtained.

Existence of Alkali Protein Compounds in Blood.—The concentrations of chlorine and bicarbonate have been repeatedly determined for normal serum and plasma by many investigators. We have tabulated some of these results in Table V in grams, gram equivalents, and their acid equivalent expressed as cc. of 0.1 N acid per liter. The concentrations of chlorine and bicarbonate in corpuscles have been determined by Fridericia (20). Means (21) and his collaborators have also studied the distribution of bicarbonate between the corpuscles and plasma of normal adults. Some of these determinations are given in Tables V and VI. The value for the concentration of inorganic phosphorus in the serum represents the average of a large number of determinations by ourselves. We have accepted Bloor's statement (22) that the inorganic phosphorus of corpuscles is about twice that of serum.[2] De Boer (23) found the concentration of sulfate in plasma to be 0.002 M. The figures recorded for the concentration of sodium, potassium, calcium, and magnesium in corpuscles and serum are the averages of all the determinations which we have made.

If one calculates the concentrations of acid and basic equivalents found in serum and corpuscles, it is found that in each case there is an excess of base of about 16 per cent. Since the pH of normal blood is about 7.35, this base cannot be free. In considering substances that occur in normal blood and might bind fixed base one thinks of (a) proteins functioning as acids (Loeb, 24) and (b) organic acids, including lactic acid and amino-acids. In a recent review Van Slyke (25) states:

[2] This statement has recently been challenged by Zucker and Gutman, who maintained that the inorganic phosphorus concentration is the same both inside and outside the red blood cells (Zucker, T. F., and Gutman, M. B., *Proc. Soc. Exp. Biol. and Med.*, 1921–22, xix, 169).

". . . . it is practically certain that it (blood) contains no substances in considerable amount of which we do not have at least sufficient knowledge to tell whether or not they can act as buffers, i.e., whether or not they are salts of weak acids or bases. An examination of the constituents (of blood) reveals among those present in amounts sufficient to have significant effect only the proteins, the bicarbonate, and the phosphate, which can be expected to act as CO_2 carrying buffers."

These and chlorine, therefore, would represent the base-binding substances of blood.

TABLE V.

Concentration of Basic and Acid Radicles in Serum.

	Per liter.	Gram equivalents per liter.	0.1 N base.
	Basic radicles.		
	gm.		*cc.*
Na...............	3.350	0.1460	1,460
K...............	0.200	0.0050	50
Ca...............	0.100	0.0050	50
Mg..............	0.030	0.0025	25
Total...........		0.1585	1,585
	Acid radicles.		
−Cl.............	3.600	0.1010	1,010
−HCO$_3$..........	1.630	0.0267	267
−HPO$_4$..........	0.092	0.0010*	18
−SO$_4$............	0.192	0.0040	40
Total..........		0.1327	1,335
Excess of base....		0.0258	250

Percentage of base not combined with acid radicles, 16 per cent.

  * This figure is really the molar concentration of HPO_4 rather than the equivalent. The difference, however, is so small as to be negligible. The equivalent of 0.1 N base has been calculated on the basis of what the ratio would be for $\dfrac{N_2HPO_4}{NaH_2PO_4}$ at pH 7.35.

According to Campbell and Poulton (26) the isoelectric point of hemoglobin is at pH 6.98. Michaelis (27) gives the isoelectric point of serum globulin as pH 5.5 and that of serum albumin as pH 4.7. The pH of normal serum is 7.35 and that of corpuscles

differs probably only slightly from this figure (28). Loeb (24) has demonstrated that at a pH greater than that of their isoelectric point proteins function as acids forming readily dissociable salts with univalent cations. These facts make the existence of protein cation compounds in serum and corpuscles highly probable.

Bloor (22) has shown that the so called undetermined phosphorus in the corpuscles may be very high. This fraction he considers as possibly an organic acid. Since we know practically nothing as to its nature, it is idle to speculate as to the possibility of its

TABLE VI.

Concentration of Basic and Acid Radicles in Corpuscles.

	Per liter.	Gram equivalents per liter.	0.1 N base.
Basic radicles.			
	gm.		*cc.*
K................	4.280	0.1097	1,097
Mg...............	0.050	0.0040	40
Total..........		0.1137	1,137
Acid radicles.			
—HCO_3..........	1.680	0.0276	276
—Cl..............	2.230	0.0628	628
—HPO_4..........	0.182	0.0020	36
Total..........		0.0924	940
Excess of base....		0.0213	197

Per cent of base not combined with acid radicles, 17 per cent.

forming any compounds with potassium. Ryffel (29) has shown that even normal blood may contain 0.012 per cent lactic acid. Nevertheless, the ease with which this acid is formed *in vitro* in biological material containing sugar raises the question of its actual existence in the circulation under normal conditions. Amino-acids might possibly bind 20 cc. of 0.1 N base in the serum and 60 cc. in the corpuscles (30).

We may conclude, therefore, that there are other substances in normal serum and corpuscles, beside the well known anions,

which bind base. These substances are probably for the most part proteins functioning as acids.

Total Available Fixed Base of Blood.

Table V shows that the sodium content of 1 liter of serum is equal to 1,460 cc. of 0.1 N base. The total available base of 1 liter of serum is equal to 1,585 cc. of 0.1 N base; *i.e.*, sodium represents 92 per cent of the mineral base of serum. The remaining 8 per cent is represented by calcium, magnesium, and potassium. It has been shown elsewhere that the magnesium concentration of serum varies but little with normal individuals, as well as with those suffering from a variety of pathological conditions. Hence for practical purposes the concentration of this element may be considered as a constant. The concentration of calcium is likewise a fixed quantity except with nephritis in adults and tetany in children (18). Here the calcium concentration of the serum is rarely reduced below 5 mg. per 100 cc. of serum. Furthermore, the potassium concentration is usually increased under the same circumstances (31). The increase of potassium is usually compensated for wholly or in part by a decrease of the calcium concentration. A decrease of the calcium concentration to 5 mg. reduces the figure for fixed base by an amount equal to 25 cc. of 0.1 N base, while an increase of potassium to 30 mg., the maximum which we have found in any case, corresponds likewise to an increase of fixed base of 25 cc. of 0.1 N base so that if we assume the concentration of K, Ca, and Mg as unchanged and add this value expressed as 0.1 N base to the figure, expressed in similar terms, . obtained by actually determining the concentration of sodium in serum we obtain, within ± 5 per cent, a measure of the total fixed base of the serum. The only element whose concentration must be determined is sodium and this can be done on 1 to 2 cc. of serum with an error that does not exceed ± 3 per cent. In a similar manner the total fixed base of corpuscles can be determined by finding the potassium concentration of whole blood, the proportion of corpuscles to serum, and assuming the K concentration of serum as 20 mg. per 100 cc.

CONCLUSIONS.

1. The corpuscles of human blood do not contain appreciable amounts of sodium or calcium.

2. The average concentration of potassium per 100 cc. of corpuscles found in thirteen normal adults was 428 mg.

3. The concentration of magnesium in whole blood is slightly higher than that of serum.

4. The extent to which the concentration of sodium, potassium, calcium, and magnesium in whole blood and corpuscles may vary is indicated in the tables.

5. Evidence is presented showing that there is an excess of about 16 per cent of basic radicles over the well known acid radicles in both serum and corpuscles. It is likely that the excess is in combination with proteins.

6. Sodium represents about 92 per cent of the fixed base of serum; potassium, practically all that of corpuscles.

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A STUDY OF CERTAIN PROTEIN PRECIPITANTS.

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Analytical methods for the separation of proteins from their split-products are based almost entirely on the greater ease with which the proteins, as compared with their products, are thrown out of solution by various coagulants and precipitants. Some of the latter agents do not precipitate all proteins, while some of them are known to precipitate certain proteolytic products; *e.g.*, heat does not coagulate gelatin, alumina cream does not precipitate hemoglobin (1); saturation with ammonium sulfate precipitates not only proteins but also some of the higher albumoses. The various precipitants are used more or less blindly in analyses of complex mixtures such as blood and protein digests.

The present paper reports the results of an attempt to ascertain empirically but somewhat more definitely the manner in which some of these precipitants act towards the proteins and protein derivatives of blood, and of Witte's peptone as a representative mixture of intermediate products. The relative proportions of total nitrogen precipitated, and of the total nitrogen, amino nitrogen, and peptide-bound nitrogen in the filtrates have been studied with seven precipitants. The latter have been applied to blood, peptone solutions, blood plus peptone, and blood plus amino-acids. In order to simplify the amino nitrogen determinations in blood filtrates the blood urea was destroyed with urease before the precipitants were used, and the ammonia formed was removed after precipitation by vacuum distillation of the filtrates.

EXPERIMENTAL.

Preparation of Blood.—1 liter samples of ox blood were collected in large bottles containing 5 gm. each of potassium oxalate. 5 gm. of Squibb's urease were added, and the blood was allowed

to stand 1 hour at room temperature. 1 per cent phenol was added and the blood was kept in the ice box, whence portions were removed as needed.

Preparation of Witte's Peptone Solution.—20 gm. of Witte's peptone were dissolved in water and diluted to a volume of 500 cc. The pH was adjusted to 7.4 and the solution was filtered. In the precipitation experiments this solution was treated as described for blood, except that the preliminary urease treatment was omitted.

Precipitation Methods.—The following methods for precipitating proteins were applied to both ox blood and Witte's peptone:

1. Colloidal Iron and Heat (2).—Before using colloidal iron for precipitating the proteins from whole blood a preliminary test was made to determine the amount of iron required. The procedure was that previously employed by Van Slyke, Vinograd-Villchur, and Losee (2) for plasma, except that we have used sodium sulfate as electrolyte instead of magnesium sulfate. Merck's dialyzed iron (5 per cent Fe_2O_3) and a 20 per cent solution of sodium sulfate were used. The procedure was the following: Into beakers were measured 20 cc. portions of water and 2 cc. portions of ox.blood. The contents were heated to boiling and colloidal iron solution was added drop by drop in the amounts designated in Table I. After a few seconds boiling the sodium sulfate solution was added as indicated in the table, and the whole was thrown onto a folded filter. The results are given in Table I.

The proportions used in No. 3 seemed satisfactory for small amounts of blood, but where large volumes were used a slightly cloudy filtrate was obtained. The larger proportions of colloidal iron indicated by No. 2 were apparently required for complete precipitation.

The colloidal iron was used in routine experiments as follows: In a large beaker were mixed 300 cc. of water and 50 cc. of the Witte's peptone solution, or of ox blood which had been treated with urease as described. The mixture was heated to boiling, then 50 cc. of colloidal iron were added drop by drop with stirring. The boiling was continued for a few seconds, while 25 cc. of 20 per cent sodium sulfate were added. The mixture was allowed to cool, then washed into a 500 cc. volumetric flask, and diluted to the mark. It was filtered through a dry folded filter; 250 cc. of the filtrate were measured into a distilling flask, made alkaline to phenolphthalein with sodium hydroxide solution, and concentrated *in vacuo* to about 10 cc.

The residue was neutralized with acetic acid, and diluted to 25 cc. This solution was analyzed in the following manner.

(a) *Total Nitrogen.*—5 cc. portions were analyzed by macro Kjeldahl, using 0.02 N acid and alkali for titration.

(b) *Amino Nitrogen.*—2 cc. portions were analyzed according to Van Slyke (3).

(c) *Peptide Nitrogen.*—To 5 cc. in a hard glass test-tube were added 5 cc. of concentrated hydrochloric acid, the tube was covered with an inverted short, wide tube, and heated for 24 hours at 100° in the steam bath. The contents were then washed into a glass evaporating dish and concentrated almost, but not quite, to dryness. The concentrated filtrates were neutralized to alizarin with 40 per cent sodium hydroxide solution and diluted to 10 cc. 2 cc. were used for amino nitrogen determinations.

TABLE I.

Behavior of Colloidal Iron as Precipitant of Whole Blood.

No.	Ox blood.	Colloidal iron solution (5 per cent Fe₂O₃).	Sodium sulfate, 20 per cent solution.	Remarks.
	cc.	cc.	cc.	
1	2	3	1.5	Filters water-clear and rapidly, but precipitate very bulky.
2	2	2	1.0	Filters water-clear and rapidly, precipitate less bulky.
3	2	1.5	0.75	Filters water-clear and rapidly, precipitate slightly less.
4	2	1.0	0.5	Filtrate yellow and cloudy, precipitate much less.

2. Tungstic Acid.—The technique followed was essentially that of Folin and Wu (4).

50 cc. of ox blood or peptone solution were measured into a 500 cc. volumetric flask to which were added 300 cc. of water and 50 cc. of a 10 per cent sodium tungstate solution,[1] and the contents were well mixed. 50 cc. of 2/3 N sulfuric acid were added, the contents were again mixed, were diluted to volume, shaken several times, and after 5 or 10 minutes were filtered through a dry folded filter. Of the filtrate 250 cc. were treated exactly as described under "Colloidal iron."

The final concentration of sodium tungstate is 1 gm. per 100 cc. of final mixture, and it is indicated as "1 per cent tungstate" in Table III.

[1] Primos Chemical Company product.

In the experiments indicated in Table III as "2 per cent tungstate" the conditions were the same, except that twice as much of both tungstate and sulfuric acid were used.

In a separate experiment with Witte's peptone, of which the results are given in Table VI, only one-fifth the above amount of peptone was used, the other details being the same.

3. Trichloroacetic Acid (5).—Ox blood and Witte's peptone were precipitated in 2.5, 5, and 10 per cent trichloroacetic acid.

2.5 Per Cent Trichloroacetic Acid.—50 cc. of blood or peptone solution were diluted with 200 cc. of distilled water, were well mixed, then diluted gradually and with constant shaking, to a volume of 500 cc. with 5 per cent trichloroacetic acid. The mixture was allowed to stand 30 minutes, and was then filtered through a dry folded filter. Of the filtrate 250 cc. were measured into a large beaker and boiled over a free flame for 15 minutes to decompose the bulk of the trichloroacetic acid ($CCl_3COOH = CHCl_3 + CO_2$). The solution was then made alkaline to phenolphthalein with a few drops of sodium hydroxide solution, was concentrated *in vacuo*, and was treated as described under "Colloidal iron."

5 Per Cent Trichloroacetic Acid.—50 cc. of blood or peptone solution were treated as above, except that 10 per cent trichloroacetic acid solution was added instead of 5 per cent. The mixture was allowed to stand 20 minutes. Of the filtrate, 250 cc. were diluted with an equal volume of water, in order to reduce the trichloroacetic acid concentration to 2.5 per cent; since with 5 per cent a slight but measurable hydrolysis of intermediate products may occur when the solution is boiled to decompose the acid. After the dilution the filtrate was boiled and treated like the 2.5 per cent filtrate.

10 Per Cent Trichloroacetic Acid.—50 cc. of blood or peptone solution were treated as above, except that 20 per cent trichloroacetic acid solution was added instead of 5 or 10 per cent. The mixture was allowed to stand 10 minutes. Of the filtrate 250 cc. were diluted 4-fold, and the procedure continued as above.

4. Ethyl Alcohol.—50 cc. of ox blood or peptone solution were diluted to 500 cc. with 95 per cent ethyl alcohol, allowed to stand 24 hours, and then filtered through a dry folded filter. To the filtrate 0.5 cc. of saturated alcoholic solution of zinc chloride was added to precipitate the last traces of protein (6). The solution was well mixed, allowed to stand for a few moments, and again filtered. 250 cc. of the filtrate were made alkaline with sodium hydroxide and concentrated *in vacuo* to a small volume. A little water was added and the solution was again concentrated to drive off the last traces of alcohol, so that the latter would not interfere with the subsequent amino nitrogen determination. The solution was then analyzed as described under "Colloidal iron."

5. Metaphosphoric Acid (7).—The metaphosphoric acid was prepared according to the method of Folin (8) and a 25 per cent solution was made

TABLE II.

Properties of Precipitates and Filtrates Obtained with Different Blood Precipitants.

Precipitant.	Relative volume of precipitate.	Appearance of filtrate.	Rate of filtration.	Volume of filtrate.	pH of filtrate.
				cc.	
Colloidal iron.	Very bulky.	Water-clear.	Rapid.	285	6.4
Tungstic acid.	Largest.	" "	Slowest.	340	5.1
Trichloroacetic acid.					
2.5 per cent.	Very bulky.	Water-clear.	Moderately rapid.	395	1.0
5.0 " "	Less bulky.	" "	Very rapid.	435	1.0
10.0 " "	Very small.	" "	Most rapid.	447	1.0
Alcohol.	Very bulky.	Yellow.	Moderate.	370	6.0
Metaphosphoric acid.	Moderately bulky.	Water-clear.	Very slow but faster than tungstic acid.	405	2.1
Picric acid.	Small.	Clear.	Moderately rapid.	430	2.2
Mercuric chloride.	Bulky.	Water-clear.	" "	225	4.4
				(Total 300)	

up just before using. Into a 500 cc. volumetric flask were measured 200 cc. of water, 50 cc. of ox blood, or of peptone solution, and 30 cc. of the 25 per cent solution of metaphosphoric acid. The contents were well mixed and allowed to stand 1 hour, then diluted to volume with water and filtered through a dry folded filter. The remaining procedure was the same as that described under "Collodial iron."

TABLE III.

Precipitations of Witte's Peptone.

Precipitant.	Peptone in 100 cc. of precipitation mixture.	Peptone N in 100 cc. of precipitate mixture.	Percentage of original peptone N in filtrate as				Volume of filtrate from 100 cc. of mixture.	pH of filtrate.
			Total filtrate N.	Amino N.	Peptide N.	Undetermined N.		
	gm.	gm.	per cent	per cent	per cent	per cent	cc.	
None...................	0.400	0.0584	100.0	10.4	62.5	27.1	100	7.4
Trichloroacetic acid.								
2.5 per cent.........	0.400	0.0584	85.4	10.5	52.3	22.6	91	> 1
5 " ".........	0.080	0.0117		10.5	51.5			
5 " ".........	0.400	0.0584	77.9	9.7	46.3	21.9	92	> 1
10 " ".........	0.400	0.0584	62.5	9.4	38.1	14.9	94	> 1
HPO_3...................	0.400	0.0584	67.8	8.3	40.4	19.1	84	1.8
$HgCl_2$...................	0.400	0.0584	71.2	8.2	38.9	24.3	83	4.7
Colloidal iron...........	0.400	0.0584	55.2	7.0	29.7	18.5	66	3.6
Picric acid..............	0.400	0.0584		5.3	19.0		80	
Alcohol.................	0.400	0.0584	29.4	4.8	21.3	4.6	88	5.7
Tungstate.								
1 per cent...........	0.080	0.0117		5.3	26.7			
2 " "...........	0.080	0.0117		5.3	27.9			
2 " "...........	0.400	0.0584		4.6	17.6			
1 " "...........	0.400	0.0584	26.7	4.0	16.2	6.5	89	2.8

6. Picric Acid.—50 cc. of blood were diluted to 500 cc. with saturated aqueous picric acid solution, allowed to stand 25 minutes, and filtered. 250 cc. of the filtrate were treated as described under "Collodial iron," the final dilution being to 50 cc. instead of 25, in order to avoid separation of an inconvenient bulk of picrate crystals.

7. Mercuric Chloride.—This precipitant was used essentially according to the technique of Gettler and Baker (9). Into a 500 cc. Erlenmeyer flask were measured 50 cc. of blood, or peptone solution, 50 cc. of water, 100 cc. of 5 per cent hydrochloric acid, and 100 cc. of 5 per cent

mercuric chloride, making a total volume of 300 cc. The solution was well mixed and filtered. 150 cc. were treated with hydrogen sulfide, and the mercuric sulfide was filtered and washed. The combined filtrate and wash water were concentrated *in vacuo* to remove hydrogen sulfide. The contents of the flask were diluted with water, made alkaline to phenolphthalein with sodium hydroxide solution, and the procedure was continued as described under "Colloidal iron."
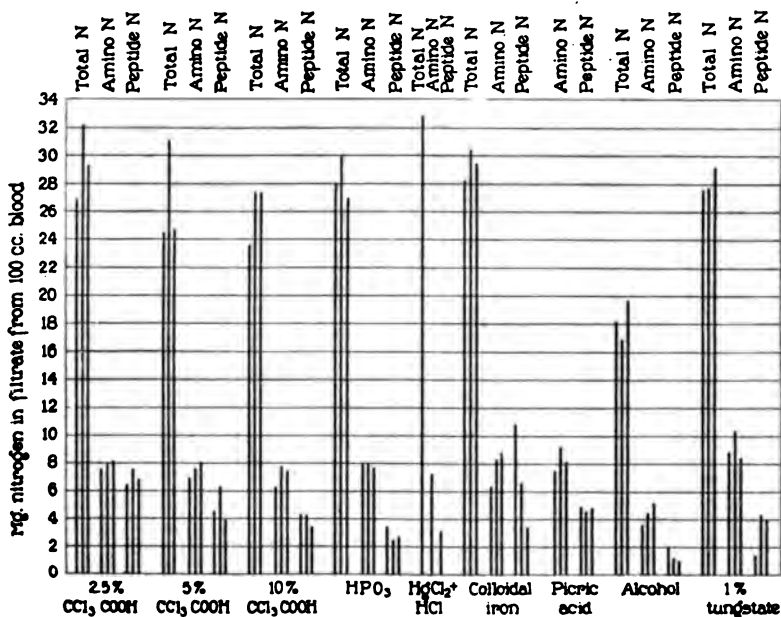
Fig. 1. Nitrogen in the filtrates from three samples of ox blood. The results from Bloods 1, 2, and 3, respectively, are indicated by three lines in order from left to right, in the case of each precipitant, except HgCl₂, which was tested only with Blood 2. For picric acid the total nitrogen figures are omitted, since the nitrogen content of the precipitant renders its filtrates unsuited for the Kjeldahl estimation.

The results obtained with three samples of ox blood are shown by Fig. 1, those with Witte's peptone by Fig. 2 and Table III. The figures for total nitrogen in blood filtrates were obtained after the urea had been removed, and therefore represent the non-protein, non-urea nitrogen.

Amino-Acids Added to Blood.—A solution of mixed monoamino-acids made from the phosphotungstic acid filtrate from hydrolyzed

casein was added to blood, so that each 100 cc. of ox blood contained an additional 22 mg. of amino nitrogen. The results of this experiment are shown in Table IV.

Precipitation of Witte's Peptone Added to Blood.—To 50 cc. portions of blood which had been treated with urease, 10 cc. portions of 4 per cent Witte's peptone solution, in which the pH had been adjusted to 7.4 were added. The proteins were immediately precipitated by the tungstic acid and 5 per cent trichloroacetic acid methods. The peptone solution was not permitted
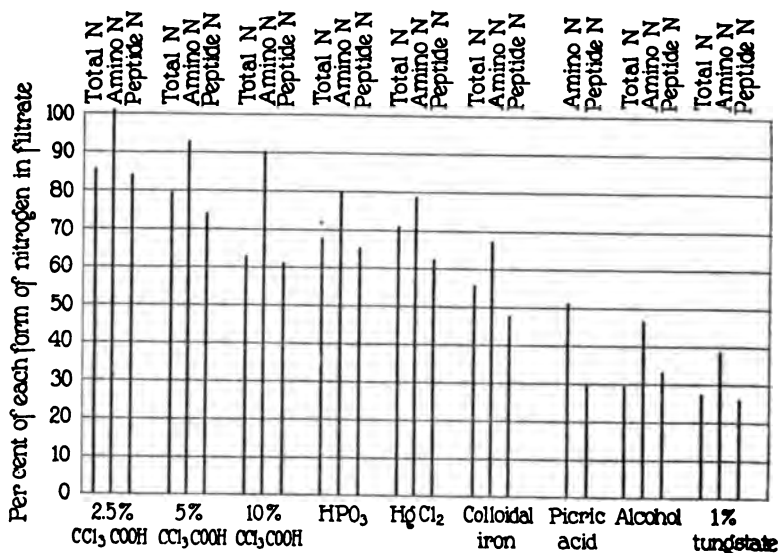
FIG. 2. Results with Witte's peptone.

to stand in contact with the urease, as the urease contains a peptolytic enzyme which rapidly splits the peptone, and thereby increases the amino nitrogen. The results of the experiment, shown in Table VI, indicate that the presence of blood does not affect the manner in which these two precipitants act on the intermediate products contained in Witte's peptone.

Hydrolytic Effect of Boiling Trichloroacetic Acid on Unprecipitated Intermediate Protein Products.—The trichloroacetic acid filtrates are boiled to decompose the acid, which when heated splits into $CHCl_3$ and CO_2. As it was uncertain whether decom-

TABLE IV.

Recovery of Amino-Acids Added to Blood.

Precipitant.	Amino nitrogen per 100 cc.				Peptide nitrogen per 100 cc.			
	Blood.	Blood + amino-acids.	Recovered.*	Added.	Blood.	Blood + amino-acids.	Recovered.*	Added.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Trichloroacetic acid, 2.5 per cent.................	9.15	30.89			8.92	8.71		
	9.82	30.75			9.83	10.39		
Average...................	9.49	30.82	21.18	22.00	9.38	9.55	+0.17	1.0
Trichloroacetic acid, 5 per cent.................	8.48	32.50			5.35	4.20		
	8.80	30.05			6.16	7.25		
Average...................	8.64	31.28	22.64	22.00	5.76	5.73	−0.03	1.0
Trichloroacetic acid, 10 per cent.................	8.00	28.95			5.09	7.95		
	8.08	29.00			4.63	8.10		
Average...................	8.04	28.98	20.94	22.00	4.86	8.03	+3.17	1.0
Metaphosphoric acid.........	8.60	27.83			4.20	9.07		
	8.12	28.04			4.85	9.51		
Average...................	8.36	27.94	19.58	22.00	4.53	9.29	+4.76	1.0
Colloidal iron...............	9.45	31.35			6.81	4.50		
	9.54	31.05			10.07	2.95		
	8.68	30.65			6.68	3.25		
Average...................	9.22	31.02	21.80	22.00	7.85	3.56	−4.29	1.0

* The figures in this column are calculated as the difference between the average figures for blood plus amino-acids and for blood alone.

The duplicate figures are from the filtrates of different precipitations and themselves represent averages obtained from aliquot parts of the same filtrate. The differences in the results from each aliquot were relatively negligible (see Table VII). Differences in this table exceeding 0.3 gm. of total or of amino + peptide N, or 0.2 mg. of amino N, are due to failure to obtain exactly identical amounts in duplicate filtrates, not to errors in the final analyses.

TABLE IV—*Concluded*.

Precipitant.	Amino nitrogen per 100 cc.				Peptide nitrogen per 100 cc.			
	Blood.	Blood + amino acids.	Recovered.*	Added.	Blood.	Blood + amino acids.	Recovered.*	Added.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Picric acid..................	9.23	30.40			4.27	6.34		
	9.29	30.85			5.19	6.75		
Average...................	9.26	30.63	21.37	22.00	4.73	6.55	+1.82	1.0
Alcohol....................	5.44	21.36			1.37	1.09		
	5.84	21.85				1.58		
Average...................	5.64	21.61	15.97	22.00		1.34	−0.03	1.0
Tungstic acid...............	9.63	31.22			5.03	4.78		
	9.98	31.25			3.52	5.60		
Average...................	9.81	31.24	21.43	22.00	4.28	5.19	+0.91	1.0

position occurs before the acid exerts an appreciable hydrolytic effect on such intermediate products as are in the filtrate, the following experiment was performed in order to test the point. The experiment was made with the filtrate from Witte's peptone rather than from blood, because the intermediate products are much more abundant in the peptone filtrate.

25 cc. portions of a 20 per cent solution of Witte's peptone were precipitated with equal volumes of 5, 10, and 20 per cent trichloroacetic acid. 3 cc. of each filtrate were neutralized with sodium hydroxide and diluted to a volume of 10 cc. 30 cc. of each filtrate were boiled in an open beaker over a free flame for 15 minutes to decompose the trichloroacetic acid, and were then diluted to 100 cc.

The amino nitrogen contents of the solutions were determined. The results as recorded in Table V showed no measurable hydrolysis as the result of boiling the peptone with trichloroacetic acid in a concentration of 2.5 per cent, but did show measurable hydrolysis by 5 and 10 per cent trichloroacetic acid.

Effect of Precipitation Time on the 5 Per Cent Trichloroacetic Acid Method.—In order to discover whether long standing after

TABLE V.

Precipitation of Blood Plus One-Fifth Its Volume of 4 Per Cent Witte's Peptone.

Method.	Amino nitrogen.				Peptide nitrogen.			
	In filtrate from 100 cc. blood.			In filtrate of peptone precipitated in absence of blood.*	In filtrate from 100 cc. blood.			In filtrate of peptone precipitated in absence of blood.*
	Blood.	Blood + 1/5 volume 4 per cent peptone.	Recovered peptone amino N.		Blood.	Blood + 1/5 volume 4 per cent peptone.	Recovered peptide N of peptone.	
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Trichloroacetic acid, 5 per cent..	7.23	20.33			5.42	64.47		
	7.23	20.73			5.30	62.97		
Average.........	7.23	20.53	13.30	12.65	5.36	63.72	58.36	60.10
Tungstate, 1 per cent.............	10.24	16.80			9.69	39.40		
	10.38	16.85			7.54	38.95		
Average.........	10.31	16.82	6.51	6.20	8.61	39.17	30.56	31.10

* These figures are calculated from Table III.

TABLE VI.

Hydrolytic Effect of Boiling Filtrate from Peptone Solution with 2.5, 5, and 10 Per Cent Trichloroacetic Acid.

Concentration of trichloro-acetic acid.	Amino N per gram of peptone.			
	Filtrate not boiled.	Filtrate boiled 15 minutes.	Increase.	
per cent	mg.	mg.	mg.	per cent
2.5	13.66	13.60	0.00	0.0
5.0	12.53	13.17	0.64	5.1
10.0	10.16	11.45	1.29	12.7

precipitation with 5 per cent trichloroacetic acid altered the results, the effect was tested both on blood and on Witte's peptone solution, the mixtures being allowed to stand 15 minutes and 24 hours,

respectively, before filtration. The results are shown in Table
VII. A very slight transformation of peptide to amino nitrogen
may have occurred during the longer period, but the change
hardly exceeds the experimental error.

Constancy of Results by the Trichloroacetic Acid Method.—In
order to determine the limit of constancy in this method, several
precipitations of the same blood were made with 2.5 and 5 per
cent trichloroacetic acid. The results are shown in Table VIII.

TABLE VII.

Effect of Precipitation Time with 5 Per Cent Trichloroacetic Acid.

Solution.	Precipitation time.	N in filtrate from 100 cc. solution.	
		Amino N.	Amino N + peptide N.
	hrs.	*mg.*	*mg.*
Witte's peptone, 4 per cent........	½	64.8	335
		64.2	335
" " 4 " "	24	66.4	333
		64.5	335
Ox blood.........................	½	7.93	12.54
		7.99	12.95
" "	24	8.15	12.20
		8.50	13.18

DISCUSSION OF RESULTS.

Results with Peptone.—From the results with Witte's peptone
it appears that tungstic acid and picric acid are distinguished by
the relative completeness with which they precipitate protein
intermediate products, without precipitating amino-acids. Tri-
chloroacetic acid on the other hand, particularly in solutions more
dilute than 5 per cent, permitted nearly all of these products to
pass into the filtrate.

It appears, therefore, that trichloroacetic acid is especially
fitted for use with solutions of partially digested proteins when it
is desired to remove the proteins, and to regain in their filtrates
not only the amino-acids, but also a maximum proportion of the
intermediate products such as "albumoses" and "peptones."
Tungstic and picric acids appear better fitted for experiments in
which it is desired to precipitate the intermediate products as
completely as possible.

TABLE VIII.

Constancy of Results with Ox Blood by Precipitation with 2.5 and 5 Per Cent Trichloroacetic Acid.

Precipitant.	N in 100 cc. blood.				Deviation from average of 4 filtrates.			
	Total N.*	Amino N.	Amino N + peptide N.	Peptide N.	Total N.	Amino N.	Amino N + peptide N.	Peptide N.
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Trichloroacetic acid, 2.5 per cent............	31.14 30.75	9.99 10.09	18.68 18.46					
Average................	30.95	10.04	18.57	8.53	+0.10	+0.26	−0.48	−0.74
Trichloroacetic acid, 2.5 per cent	31.30 31.58	9.81 9.81	18.94 19.16					
Average................	31.44	9.81	19.05	9.24	+0.59	+0.03	±0.00	−0.03
Trichloroacetic acid, 2.5 per cent............	30.47 30.40	9.64 9.56	19.50 19.32					
Average................	30.44	9.60	19.41	9.81	−0.41	−0.18	+0.36	+0.54
Trichloroacetic acid, 2.5 per cent............	30.20 30.90	9.64 9.72	19.16 19.13					
Average................	30.55	9.68	19.16	9.48	−0.30	−0.10	+0.11	+0.21
Average of 4 filtrates	30.85	9.78	19.05	9.27				
Trichloroacetic acid, 5 per cent..............	26.60 26.78	9.67 9.52	15.45 15.32					
Average................	26.69	9.60	15.39	5.79	−0.74	+0.08	+0.48	+0.40
Trichloroacetic acid, 5 per cent	27.59 27.59	9.64 9.63	14.27 14.36					
Average................	27.59	9.64	14.32	4.68	+0.16	+0.12	−0.59	−0.71
Trichloroacetic acid, 5 per cent	27.48 27.30	9.44 9.33	14.59 14.86					
Average................	27.39	9.39	14.73	5.34	−0.04	−0.13	−0.18	−0.05
Trichloroacetic acid, 5 per cent	27.88 28.20	9.51 9.36	15.25 15.14					
Average................	28.04	9.44	15.20	5.76	+0.61	−0.08	+0.29	+0.37
Average of 4 filtrates	27.43	9.52	14.91	5.39				

* The total nitrogen figures represent the total nitrogen of the filtrates from blood from which the urea had previously been removed, and represent, therefore, the non-protein, non-urea nitrogen.

Alcohol behaves toward Witte's peptone like tungstic and picric acids, but for reasons discussed below, is not a desirable precipitant for quantitative work.

Metaphosphoric acid, colloidal iron, and mercuric chloride are intermediate between trichloroacetic acid and tungstic acid in the completeness with which they precipitate the intermediate products of Witte's peptone.

Results with Blood.—The average figures obtained with the different precipitants are given in Table IX.

All the precipitants used appear to remove the blood proteins completely. The completeness of the removal is indicated by the

TABLE IX.

Average of Results Obtained with Three Ox Bloods.

Precipitant.	Nitrogen per 100 cc. of blood.		
	Total non-protein, non-urea N.	Amino N.	Peptide N.
	mg.	*mg.*	*mg.*
Tungstic acid.............................	28.1	9.2	4.1
Picric acid................................	*	8.3	4.6
Metaphosphoric acid.......................	28.3	7.9	3.9
2.5 per cent trichloroacetic acid............	28.8	7.9	7.0
Colloidal iron.............................	29.4	7.8	†
5 per cent trichloroacetic acid..............	26.7	7.5	4.9
10 per cent trichloroacetic acid.............	26.1	7.1	4.6
Alcohol...................................	18.2	4.9	1.4

* Not determined because of nitrogen content of precipitant.

† Not averaged because of inconsistency of results.

lack of high and irregular figures for the total filtrate nitrogen, and in particular for the peptide nitrogen, such as would have been obtained had even slight proportions of the relatively immense amounts of protein nitrogen present escaped precipitation.

Of the amino nitrogen naturally present in blood, all of the precipitants except alcohol permitted similar though not exactly equal amounts (8 ± 1 mg. per 100 cc.) to pass into the filtrates. In the filtrates from alcohol only about two-thirds as much amino nitrogen was found as in the filtrates from the precipitants used in aqueous solution. Mixed monoamino-acids from hydrolyzed casein added to blood were recovered with approximate complete-

ness in all the filtrates except those from alcohol and metaphosphoric acid (Table IV), from which were recovered 73 and 89 per cent, respectively. Apparently when alcohol is used as a precipitant of the blood, about 30 per cent of the free amino-acids present are adsorbed by the coagulated proteins.[2] Our findings in this respect agree with those of Bock (13).

From the peptide nitrogen data it is evident that, unlike Witte's peptone, the bloods examined contained no appreciable amounts of intermediate products precipitated by picric and tungstic acid, but not by 5 or 10 per cent trichloroacetic or metaphosphoric acid. All five of these precipitants yielded nearly the same peptide nitrogen. The bloods did, however, show 2 to 3 mg. of peptide nitrogen per 100 cc. precipitable by the above reagents, but not by 2.5 per cent trichloroacetic acid.

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12. György, P., and Zunz, E., *J. Biol. Chem.*, 1915, xxi, 511.
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[2] Van Slyke and Meyer (10) recovered relatively large amounts of added alanine almost completely from the alcoholic filtrate of dog's blood, but with the smaller concentrations of the mixed amino-acids normally present the proportion adsorbed is too great to permit quantitative recovery. The results obtained by ourselves and other authors indicate, however, that a fairly constant fraction of the total amino-acid nitrogen, *viz.* about two-thirds, is regained in the alcohol filtrate, and that this fraction is sufficiently constant to validate the conclusions drawn from comparative results in physiological experiments such as those of Van Slyke and Meyer (10), Folin (11), and Zunz (12).

THE DETERMINATION OF THE THREE DISSOCIATION CONSTANTS OF CITRIC ACID.

By A. BAIRD HASTINGS AND DONALD D. VAN SLYKE.

(From the Hospital of The Rockefeller Institute for Medical Research.)

Although the first dissociation constant of citric acid has been found by conductivity to be 8.2×10^{-4} by Walden,[1] and 8.0×10^{-4} by Walker[2] at 25°, and by pH determinations 7.9×10^{-4} by Enklaar[3] at 18°, the values of the second and third dissociation constants have not been available. From the data obtained by the electrometric titration of citric acid, and from formulas developed by Van Slyke in a previous paper,[4] we have calculated the three dissociation constants of the acid.

The electrometric titration of 0.1 M citric acid with 1.0 N NaOH was carried out in a titrating electrode vessel recently described by Hastings.[5] To check these determinations the pH values of citric acid-sodium citrate mixtures, 0.1 M with respect to the citrate radical, were also determined in a Clark cell. These determinations were carried out at $20 \pm 0.1°$. Table I and Fig. 1 show the results.

The calculations were performed in the following way. The equation for the dissociation of a weak acid in the presence of its alkali salt, at pH ranges less than 11, is No. 31 of a previous paper;[4] *viz.*,

$$K'_a = \frac{[H^+] \, (B + [H^+])}{C - (B + [H^+])},$$

where B is the amount of alkali added to the acid Ha, C is the concentration of the acid, γ the degree of dissociation of the salt

[1] Walden, P., *Z. physik. Chem.*, 1892, x, 568.
[2] Walker, J., *J. Chem. Soc.*, 1892, lxi, 708.
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[5] Hastings, A. B., *J. Biol. Chem.*, 1921, xlvi, 463.

TABLE I.

Electrometric Titration of Citric Acid with NaOH. Solutions 0.1 M with Respect to Citrate.

NaOH	pH*	[H$^+$]	NaOH	pH	[H$^+$]	NaOH	pH	[H$^+$]
mols per liter			*mols per liter*			*mols per liter*		
0.0000	2.06	8.70×10^{-3}	0.1578	4.46	3.47×10^{-5}	0.2821	6.17	6.76×10^{-7}
0.0197	2.51	3.09×10^{-3}	0.1775	4.69	2.04×10^{-5}	0.2841	6.25	5.62×10^{-7}
0.0395	2.88	1.32×10^{-3}	0.1973	4.94	1.15×10^{-5}	0.2861	6.32	4.78×10^{-7}
0.0592	3.14	7.25×10^{-4}	0.2170	5.18	6.61×10^{-6}	0.2880	6.39	4.07×10^{-7}
0.0790	3.42	3.80×10^{-4}	0.2368	5.42	3.80×10^{-6}	0.2900	6.54	2.88×10^{-7}
0.0987	3.67	2.14×10^{-4}	0.2564	5.70	1.995×10^{-6}	0.2920	6.58	2.63×10^{-7}
0.1183	3.98	1.05×10^{-4}	0.2762	6.06	8.71×10^{-7}	0.2939	6.83	1.48×10^{-7}
0.1381	4.20	6.31×10^{-5}	0.2782	6.07	8.51×10^{-7}	0.2959	7.17	6.76×10^{-8}
			0.2802	6.14	7.25×10^{-7}			

* Standard solution used for determining potential of calomel cell = 0.1 N HCl. pH assumed = 1.085, 20°. Gas chain consisted of Pt — H$_2$ — solution X — saturated KCl — HgCl$_2$ — Hg.
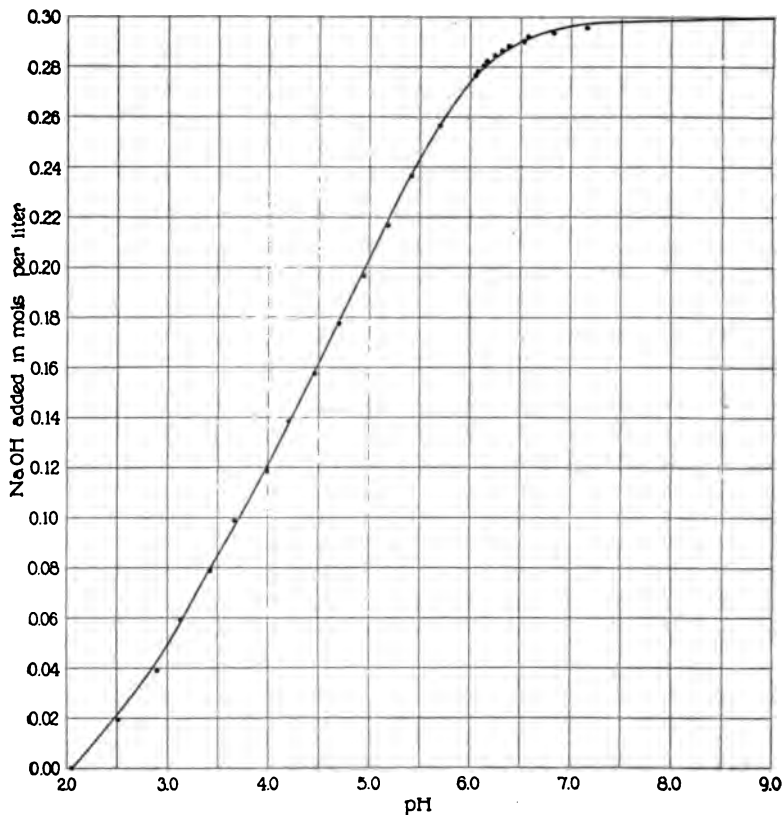
FIG. 1.

Ba into B^+ and a', and $K'_a = \dfrac{K_a}{\gamma}$. In solutions where the concentration of Ba is small, γ may be assumed to approach 1.0, and K'_a to approach K_a (this condition will be noted in Table III).

TABLE II.

Calculation of K'_1 from the Formula

$$K'_1 = \frac{[H^+]\,(B + [H^+])}{C - (B + [H^+])}$$

without Correction for B_2 and B_3.

C = 0.1000

[H$^+$]	B	B + [H$^+$]	C − (B + [H$^+$])	K$_1'$
8.7×10^{-3}	0.0000	0.0087	0.0913	8.3×10^{-4}
3.09×10^{-3}	0.0197	0.0228	0.0772	9.1×10^{-4}
1.32×10^{-3}	0.0395	0.0408	0.0592	9.1×10^{-4}
7.25×10^{-4}	0.0592	0.0599	0.0401	10.8×10^{-4}
3.80×10^{-4}	0.0790	0.0794	0.0206	14.6×10^{-4}

TABLE III.

Calculation of K'_1 after the Correction of B_1 for B_2 and B_3 by the Formulas

$$B_2 = \frac{K'_2 C}{[H^+] + K_2} \quad \text{and} \quad B_3 = \frac{K'_3 C}{[H^+] + K'_3}$$

$$K'_2 = 4.1 \times 10^{-5}$$
$$K'_3 = 3.2 \times 10^{-6}$$
$$C = 0.1000$$

[H$^+$]	Total B	B$_2$	B$_3$	B$_1$	B$_1$ + [H$^+$]	C − (B$_1$ + [H$^+$])	K$_1'$
8.7×10^{-3}	0.0000	0.0000	0.0000	0.0000	0.0087	0.0913	8.3×10^{-4}*
3.09×10^{-3}	0.0197	0.0013	0.0001	0.0183	0.0214	0.0786	8.4×10^{-4}
1.32×10^{-3}	0.0395	0.0030	0.0002	0.0363	0.0376	0.0624	7.9×10^{-4}
7.25×10^{-4}	0.0592	0.0054	0.0004	0.0534	0.0541	0.0459	8.5×10^{-4}
3.80×10^{-4}	0.0790	0.0098	0.0008	0.0684	0.0688	0.0312	8.3×10^{-4}

* The value for this first figure is really K_1 rather than K'_1, since no salt is present.

$$\text{Average } K'_1 = 8.3 \times 10^{-4}$$
$$pK'_1 = 3.08$$

The values of K'_1, K'_2, and K'_3 were estimated by successive approximations. (We shall refer to the K'_a of the most strongly dissociated acid group as K'_1, of the middle one as K'_2, and of the least dissociated as K'_3.) From the data towards the most acid

and least acid ends of the titration curve (Fig. 1), first approximations of the values of K'_1 and K'_3, respectively, were made (Tables II and IV). From the approximate values of K'_1 and K'_3 thus obtained the amounts of base, B_1 and B_3, bound by the two end-carboxyls over the middle part of the curve, were calculated by Equation 10 of the previous paper;[4] viz., $B = \dfrac{K' C}{[H^+]+K'}$. The B_1 and B_3 values thus calculated were subtracted from the total amount of NaOH added (B) to give B_2. $B_2 = B - B_1 - B_3$. From the values of B_2 thus obtained K'_2 was calculated.

TABLE IV.

Calculation of K'_2 from the Formula

$$K'_2 = \frac{[H^+][B]}{C - B}$$

without Correction for B_1 and B_3.

C = 0.1000

[H$^+$]	B	C − B	K'$_2$
2.63 × 10^{-7}	0.0920	0.0080	3.0 × 10^{-6}
2.88 × 10^{-7}	0.0900	0.0100	2.6 × 10^{-6}
4.07 × 10^{-7}	0.0880	0.0120	3.0 × 10^{-6}
4.78 × 10^{-7}	0.0861	0.0139	3.0 × 10^{-6}
5.62 × 10^{-7}	0.0841	0.0159	3.0 × 10^{-6}
6.76 × 10^{-7}	0.0821	0.0179	3.1 × 10^{-6}
7.25 × 10^{-7}	0.0802	0.0198	2.9 × 10^{-6}
8.51 × 10^{-7}	0.0782	0.0218	3.0 × 10^{-6}
8.71 × 10^{-7}	0.0762	0.0238	2.8 × 10^{-6}
1.995 × 10^{-6}	0.0564	0.0436	2.6 × 10^{-6}
3.80 × 10^{-6}	0.0368	0.0632	2.2 × 10^{-6}

From this value of K'_2 and the above mentioned first approximations of K'_2 and K'_3, B_2 and B_3 were estimated, in order to calculate the exact B_1, at the acid end of the curve by the equation $B_1 = B - B_2 - B_3$. From the B_1 values thus obtained, a series of consistent values for K'_1 was obtained (Table III).

The B_3 values at the alkaline end of the curve were then estimated as $B_3 = B - B_1 - B_2$, the B_1 and B_2 values being obtained from the K'_1 and K'_2 values, found as above described. The B_3 values thus obtained yielded a series of consistent values for K'_3 (Table V).

TABLE V.

Calculation of K_2' after the Correction of B_2 for B_1 and B_3 by the Formulas

$$B_1 = \frac{K_1'C}{[H^+] + K_1'} \text{ and } B_2 = \frac{K_2'C}{[H^+] + K_2'}$$

$$K_1' = 8.3 \times 10^{-4}$$
$$K_2' = 4.1 \times 10^{-5}$$
$$C = 0.1000$$

[H⁺]	Total B	B₁	B₂	B₃	C − B	K₃′
2.63 × 10⁻⁷	0.2920	0.1000	0.0994	0.0926	0.0074	3.3 × 10⁻⁶
2.88 × 10⁻⁷	0.2900	0.1000	0.0993	0.0907	0.0093	2.8 × 10⁻⁶
4.07 × 10⁻⁷	0.2880	0.1000	0.0991	0.0889	0.0111	3.3 × 10⁻⁶
4.78 × 10⁻⁷	0.2861	0.1000	0.0990	0.0871	0.0129	3.2 × 10⁻⁶
5.62 × 10⁻⁷	0.2841	0.1000	0.0988	0.0853	0.0147	3.3 × 10⁻⁶
6.76 × 10⁻⁷	0.2821	0.1000	0.0985	0.0836	0.0164	3.4 × 10⁻⁶
7.25 × 10⁻⁷	0.2802	0.1000	0.0983	0.0819	0.0181	3.3 × 10⁻⁶
8.51 × 10⁻⁷	0.2782	0.1000	0.0982	0.0800	0.0200	3.4 × 10⁻⁶
8.71 × 10⁻⁷	0.2762	0.1000	0.0981	0.0781	0.0219	3.1 × 10⁻⁶
1.995 × 10⁻⁶	0.2564	0.0998	0.0954	0.0612	0.0388	3.1 × 10⁻⁶
3.80 × 10⁻⁶	0.2368	0.0996	0.0917	0.0455	0.0545	3.2 × 10⁻⁶

Average $K_3' = 3.2 \times 10^{-6}$
$pK_3' = 5.49$

TABLE VI.

Calculation of K_2' from the Formula

$$K_2' = \frac{[H^+]\ [B_2]}{C - B_2}$$

where B_2 is Calculated from

$$B_2 = B - [B_1 + B_3]$$
$$K_1' = 8.3 \times 10^{-4}$$
$$K_3' = 3.2 \times 10^{-6}$$
$$C = 0.1000$$

[H⁺]	Total B	B₁	B₃	B₂	C − B₂	K′₂
3.80 × 10⁻⁴	0.0790	0.0686	0.0008	0.0096	0.0904	4.0 × 10⁻⁵
2.14 × 10⁻⁴	0.0987	0.0798	0.0015	0.0174	0.0826	4.5 × 10⁻⁵
1.05 × 10⁻⁴	0.1183	0.0888	0.0030	0.0265	0.0735	3.8 × 10⁻⁵
6.31 × 10⁻⁶	0.1381	0.0930	0.0048	0.0403	0.0597	4.3 × 10⁻⁵
3.47 × 10⁻⁶	0.1578	0.0961	0.0084	0.0533	0.0467	4.1 × 10⁻⁵
2.04 × 10⁻⁵	0.1775	0.0977	0.0135	0.0663	0.0337	4.0 × 10⁻⁵

Average $K_2' = 4.1 \times 10^{-5}$
$pK_2' = 4.39$

K'_2 was finally reestimated by using the accurate K'_1 and K'_3 values to calculate the B_1 and B_3 figures of the equation $B_2 = B - B_1 - B_3$. The B_2 values thus obtained yielded a series of consistent values for K'_2 (Table VI).

TABLE VII.

Calculation of K'_2 from the Buffer Value β_2.

$$pK'_1 = 3.08$$
$$pK'_3 = 5.49$$

pH	ΔpH	Mean pH	ΔB	$\frac{\Delta B}{\Delta pH}$	pH− pK'₁	pH− pK'₃	β_1	β_3	β_2	pH− pK'₂	pK'₂
3.69 3.95	0.26	3.82	0.02	0.0770	0.74	−1.67	0.0301	0.0046	0.0423	−0.49	4.31
3.82 4.08	0.26	3.95	0.02	0.0770	0.87	−1.54	0.0241	0.0063	0.0466	−0.40	4.35
3.95 4.22	0.27	4.09	0.02	0.0741	1.01	−1.40	0.0190	0.0083	0.0468	−0.39	4.48
4.08 4.35	0.27	4.22	0.02	0.0741	1.14	−1.27	0.0146	0.0112	0.0483	−0.37	4.59
4.22 4.48	0.26	4.35	0.02	0.0770	1.27	−1.14	0.0112	0.0147	0.0511	−0.30	4.65
4.35 4.59	0.24	4.47	0.02	0.0834	1.39	−1.02	0.0086	0.0184	0.0564	+0.10	4.37
4.48 4.72	0.24	4.60	0.02	0.0834	1.52	−0.89	0.0063	0.0233	0.0538	+0.23	4.37
4.59 4.84	0.25	4.72	0.02	0.0800	1.64	−0.77	0.0052	0.0288	0.0460	+0.42	4.30
4.72 4.97	0.25	4.85	0.02	0.0800	1.77	−0.64	0.0037	0.0345	0.0415	+0.51	4.34

K'_2 was also calculated from the buffer value $\frac{dB}{dpH}$. The total buffer value of the solution at any pH may be expressed as $\beta = \beta_1 + \beta_2 + \beta_3$. β may be evaluated from the titration curve

by calculating $\dfrac{\Delta B}{\Delta pH}$. β_1 and β_3 may be calculated[6] from K'_1 and K'_3 by Equation 35; *viz.*,

$$\beta = 2.3 \left(\frac{K' \, C \, [H^+]}{(K' + [H^+])^2} + [H^+] + [OH'] \right),$$

the [OH'] being negligible in the present case. They may also be estimated graphically by means of Fig. 9 of the previous paper.[4]
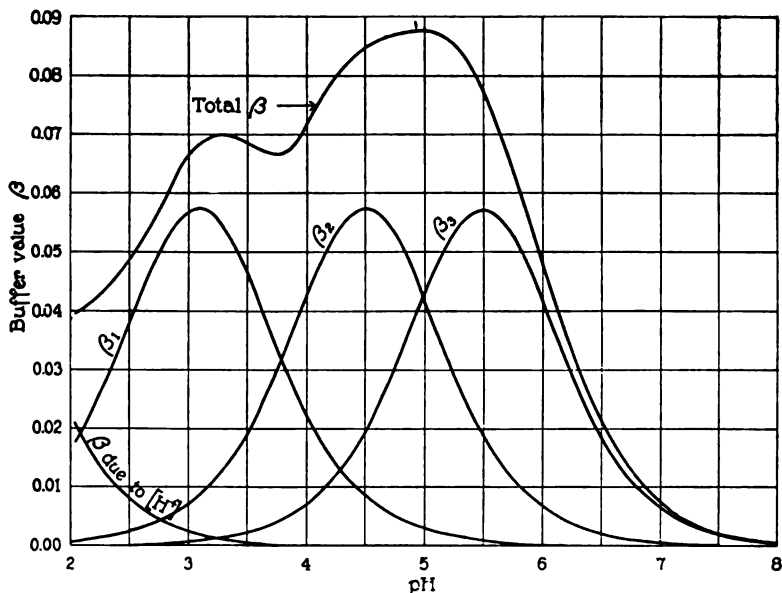
FIG. 2.

β_2 is then obtained by difference, and K'_2 calculated either from Equation 22,

$$K'_2 = [H^+] \frac{C - 0.8686 \, \beta_M \pm \sqrt{(0.8686 \, \beta_M - C)^2 - 0.756 \, \beta_M{}^2}}{0.8686 \, \beta_M}$$

or graphically, as described in the previous paper, from Fig. 9. That value of K'_2 is the correct one which is identical with the $[H^+]$ when $\beta_M = 0.575$.

[6] The equation numbers used in the present paper refer to the numbered equations in a former paper.[4]

Values of pK_2' obtained by this method are given in Table VII. The value at that point where β_2 most nearly approaches its maximum of 0.0564, is $pK' = 4.37$.   Other values range from 4.30 to 4.65.   The agreement with the first method of calculation is, we believe, sufficiently good.

The value of the buffer effect of 0.1 M citrate, estimated from the above three pK' values by graphic summation (as in[4] Fig. 8) are given in Fig. 2.

SUMMARY.

The methods for the calculation of the dissociation constants of weak polybasic acids recently outlined[4] have been applied to citric acid.   The values of the three constants have been found to be $K_1' = 8.3 \times 10^{-4}$, $K_2' = 4.1 \times 10^{-5}$, and $K_3' = 3.2 \times 10^{-6}$. The corresponding pK' values are 3.08, 4.39, and 5.49, respectively. The value of K_1' agrees approximately with that of K_1 found by other authors. [1, 2, 3]   The values of K_2' and K_3', because of the overlapping effects of the carboxyl groups, have not been accessible by previous methods of calculation.

THE EFFECT OF ETHER ANESTHESIA ON THE ACID-BASE BALANCE OF THE BLOOD.

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There has been disagreement in the literature concerning the effect of ether anesthesia on the acid-base balance. Menten and Crile (1915) reported a fall in the blood pH in rabbits. Caldwell and Cleveland (1917) found a consistent fall in the plasma bicarbonate as the result of ether anesthesia in patients. Carter (1920) found a similar fall in etherized dogs. If observations by Caldwell and Cleveland and Carter on blood bicarbonate could be linked with Menten and Crile's pH determinations on rabbits, the combined data, indicating lowered [BHCO$_3$] and lowered pH, respectively, could indicate nothing but an uncompensated alkali deficit.

However, Y. Henderson and Haggard (1918) in experiments on dogs showed that the blood alkali reserve, as measured by the CO$_2$ capacity, could be lowered by experimental hyperventilation. The latter lowers the free H$_2$CO$_3$ of the blood, with the result that as shown by Milroy (1914), an increased pH results. The results of Henderson and Haggard indicated that as a secondary effect there may also be a shift of alkali from blood to tissues, or of acid in the reverse direction. Such an effect could be explained as an attempt to lessen the increased pH resulting from abnormal lowering of H$_2$CO$_3$. The result of such a condition is indeed a lowered bicarbonate, but, it is primarily due to H$_2$CO$_3$ deficit, or CO$_2$ alkalosis (exemplified by Areas 2 and 3 of Fig. 1, Van Slyke, 1921, *b*), rather than to alkali deficit resulting from entrance of non-volatile acids (exemplified by Areas 6 and 9).

Hasselbalch and Lundsgaard (1912) had showed that merely tying rabbits down may result in a gradual fall in blood pH; so there appeared to be some reason for being uncertain that Menten and Crile's observed fall in blood pH was due to etherization. Henderson and Haggard reported no pH determinations, but their results with ether seemed most readily interpreted by the CO_2 deficit explanation.

It appeared that a decision between the two diametrically opposed interpretations of the observed fall in blood [$BHCO_3$] could be reached only by observations in which both [$BHCO_3$] and pH were determined. In a preliminary report (1920) the present authors showed that the pH falls (in agreement with Crile and Menten) whenever it undergoes any change during ether anesthesia. Fall in bicarbonate was consistently observed.

Since the appearance of the above report Collip (1920), experimenting with etherized dogs, calculated the blood pH from the CO_2 tension of the alveolar air and the CO_2 content of the blood. He found this method not entirely satisfactory, but indicative of a fall in blood pH. Recently Atkinson and Ets (1922) have again determined both CO_2 content and pH (the latter by the Dale and Evans colorimetric dialysis method) in the blood of etherized dogs, and have found a fall in both, with recovery after discontinuance of anesthesia.

In the present paper we report the results of six representative experiments. The pH changes in some cases were determined electrometrically, in others colorimetrically, and in others by calculation from the [$BHCO_3$] : [H_2CO_3] ratio obtained by equilibration with known CO_2 tensions. The bicarbonate was determined both gasometrically and by titration.

The depth and time of anesthesia have been varied. The results concerning the acid-base change have been consistent, and we have consequently felt justified in reporting in detail only a sufficient number of experiments to indicate fairly the nature of the data obtained.

Methods.

Animals.—Large dogs (10 to 15 kilos) were used so that large samples of blood (25 to 50 cc.) might be taken. The dogs were fed as usual the day preceding the experiment but received no food on the day of the experiment.

Bleeding.—The blood was drawn from the left ventricle (unless stated otherwise) through a 4 inch, 16 gauge lumbar puncture needle into a tube under oil. When oxalated blood was desired the tube was previously coated with neutral potassium oxalate to make 0.3 per cent. If defibrinated blood was wanted, the blood was defibrinated under oil by gentle stirring. Since it is usually impossible to avoid some hemolysis in oxalated plasma of dog's blood, it was decided in the later experiments to utilize the true serum of the blood as drawn, allowing coagulation to occur spontaneously while centrifuging.

In centrifuging for true plasma or true serum the blood was drawn directly into a centrifuge tube of the proper size, containing mineral oil. The glass delivery tube was withdrawn as the blood ran in, so that the tube was completely filled with the blood except for a layer of paraffin oil about 1 cm. deep. A 1-hole rubber stopper was inserted, with complete expulsion of the oil. The hole was closed with a glass plug and the tube centrifuged at once. After centrifuging, the glass plug was removed, and from a pipette oil was allowed to flow through the hole in the stopper as the stopper was removed. It is shown elsewhere that such precautions are necessary to prevent loss of CO_2 during centrifugation. The plasma or serum was then transferred without loss of CO_2 to Haldane sampling tubes over mercury or to tubes under oil.

Anesthesia.—The animal was anesthetized in all cases by the drop method with a few layers of gauze. This method was continued throughout the anesthesia unless otherwise stated.

Ventilation Rate.—Ventilation rate was measured in the control periods and during the early part of anesthesia by the use of a closely fitting, well greased rubber mask that fitted and enclosed the entire muzzle of the dog. A Y-tube close to the mask led to two 1 inch aluminum Siebe Gorman valves. The expired air was collected and measured in a 65 liter spirometer.

After the animal was anesthetized a cannula was tied in the trachea and connected to the Y-tube and valves. The intake of air was through a vessel containing gauze onto which ether could be dropped to maintain the anesthesia.

Equilibration of Blood or Serum with CO_2.—The blood was introduced into a partially evacuated tonometer which contained

the required amount of CO_2 and had an oxygen tension approximately atmospheric at 38°. The tonometer was then rotated in a water bath at 38° until equilibration was complete. The equilibration was either repeated or the CO_2 tensions corrected for the CO_2 taken up or given off by the blood. The blood was then transferred, with precautions to prevent loss of CO_2, to Haldane sampling tubes over mercury, or, under oil, to centrifuge tubes. The centrifuging was carried out with the precautions described above.

Hydrogen Ion Concentration Measurements.—Electrometric.— These determinations were made at 20° on whole blood with the Clark cell using Hasselbalch's refilling technique. Although the determinations were made with the cells present, Parsons (1919–20) has shown that the pH determined is that of the plasma.

Colorimetric.—The colorimetric pH measurements were made at room temperature with phenol red in the diluted plasma or serum by the method recently described by Cullen (1922). The correction used was − 0.34, to reduce pH colorimetrically determined at 20° to that electrometrically found at 38°. This correction may subsequently be altered, but such alteration would not affect the pH changes observed.

Analytical Methods.—The carbon dioxide determinations were made usually in duplicate on 1 cc. samples by Van Slyke's method with either the fine bore constant pressure apparatus (Van Slyke and Stadie, 1921), or the constant volume apparatus (Van Slyke, 1921, *a*). In Experiment 5, in addition to the gasometric determination of the total CO_2 content, the $BHCO_3$ was determined directly by the titration method (Van Slyke, 1922, *b*).

Calculation.

In discussion of the calculations we shall use the following abbreviations: mM. for millimolar; $[CO_2]$, $[BHCO_3]$, $[H_2CO_3]$ for mM. concentration of CO_2, $BHCO_3$, and H_2CO_3, respectively; p_{CO_2} for CO_2 tension in millimeters of mercury.

In these experiments we have obtained by analysis two or more of the following data: $[CO_2]$ of the blood, plasma, or serum, as drawn from the left ventricle or femoral artery; $[CO_2]$ of the oxygenated blood, true plasma, or serum after equilibration of the blood at 38° in tonometers at known p_{CO_2}; the pH

determined electrometrically or colorimetrically on the plasma or serum as drawn, or upon the true plasma or serum after equilibration at known p_{CO_2}. Our problem has been to determine from these data the changes that have occurred, *in vivo*, in the alkaline reserve, CO_2 tension, and pH of the blood.

In Experiments 1 to 3 we have data on the $[CO_2]$ of blood, or the plasma of blood, equilibrated at various CO_2 tensions, and the $[CO_2]$ of the arterial blood, plasma, or serum, as drawn. From the $[CO_2]$ and p_{CO_2} values determined in the equilibrated blood or its true serum, we have estimated the pH values of the blood plasma, and plotted them as abscissæ against the $[CO_2]$ values as ordinates. The resulting curves are almost exactly straight lines.[1] By interpolating the $[CO_2]$ values observed in the blood as drawn on these lines we have determined the pH of the blood as drawn. From the pH thus interpolated and the $[CO_2]$, the p_{CO_2} and $[BHCO_3]$ were calculated.

In constructing our $[CO_2]$, pH curves we have in each case drawn the mean straight line through the points determined on the equilibrated bloods. It appears that such a line compensates for errors in individual determinations and is a more accurate representation of the correct $[CO_2]$, pH curves than a broken line drawn through the individual points. The pH values corrected by means of the straight line graphs thus drawn are indicated in the tables as "Rectified pH" points. It will be noted that the

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[1] The fact that when blood pH is plotted against $[CO_2]$ straight line curves are obtained over the physiological range of CO_2 tensions was first noted by Lewis, Cotton, Barcroft, Milroy, Duften, and Parsons (1916). McLean, Murray, and Henderson (1920) found straight line curves also when they plotted pH values against $[BHCO_3]$. The approximately linear character of these curves is attributable to the, as one might say, accidental fact, that the COOH groups of hemoglobin are so arranged that the buffer value (Van Slyke, 1922, *a*) of blood over the physiological pH range is practically constant.

Both curves cannot be exactly linear, for the $[H_2CO_3]$ area which separates $[CO_2]$ from $[BHCO_3]$ is curved. However, this area above pH 7 is relatively narrow, so that it does not prevent both the $[CO_2]$ and $[BHCO_3]$ curves from approximating the linear form. Which of the two does so most closely is at present uncertain, since both appear, over the range pH 7 to 7.8 to vary from straight lines by no more than the experimental errors heretofore connected with pH and $[CO_2]$ measurements.

rectifications fall within the limit of 0.02 pH, which may be taken as the limit of experimental error.

The calculations of pH from $[CO_2]$ and p_{CO_2} values obtained from equilibration data, and of p_{CO_2} and $[BHCO_3]$ from the determined $[CO_2]$ in the blood as drawn and the pH found by graphic interpolation, were performed by means of Hasselbalch's (1917) equation, $pH = pK' + \log \dfrac{[BHCO_3]}{[H_2CO_3]}$. For the above respective calculations the equation was rearranged into the following forms by steps given in another place (Austin, Cullen, Hastings, McLean, Peters, and Van Slyke, 1922).

$$\text{For whole blood, } pH = 6.20 + \log \frac{[CO_2] - 0.0300 \, p_{CO_2}}{0.0300 \, p_{CO_2}},$$

$$p_{CO_2} = \frac{1}{0.0300} \times \frac{[CO_2]}{10^{pH - 6.20} + 1}, \; [BHCO_3] = [CO_2] \times \frac{10^{pH - 6.20}}{10^{pH - 6.20} + 1}.$$

$$\text{For plasma, } pH = 6.10 + \log \frac{[CO_2] - 0.0318 \, p_{CO_2}}{0.0308 \, p_{CO_2}},$$

$$p_{CO_2} = \frac{1}{0.0318} \times \frac{[CO_2]}{10^{pH - 6.10} + 1}, \; [BHCO_3] = [CO_2] \times \frac{10^{pH - 6.10}}{10^{pH - 6.10} + 1}.$$

The values of pK', 6.10 for plasma and 6.20 for whole blood, are the averages determined on a number of dog bloods. They may later be corrected in the second decimal places but such correction would not significantly affect the *changes* in pH, p_{CO_2}, and $[BHCO_3]$ calculated in our experiments.

In terms of millimolar concentration, $[H_2CO_3] = \dfrac{\alpha CO_2}{0.0224 \times 760} \dfrac{p_{CO_2}}{}$. This value is $0.0300 \, p_{CO_2}$ when α_{CO_2} (the solubility coefficient of CO_2) is 0.511, as in whole blood (Bohr, 1905), while it is $0.0318 \, p_{CO_2}$ when α_{CO_2} is 0.541, as in serum.

Changes in alkali reserve have been measured as changes in the bicarbonate content of the blood estimated at a given pH; *viz.*, the pH observed before ether was administered. This pH was interpolated on the pH, $[CO_2]$ graph obtained after ether, and the $[BHCO_3]$ was calculated for the point thus located. At constant pH, the change in blood $[BHCO_3]$ expresses directly the change in the excess of total base over acids other than H_2CO_3 (Van Slyke, 1921, *b*, p. 169), and because of this advantage in definiteness of interpretation, we have chosen to measure bicarbonate changes at a given pH rather than at a given p_{CO_2}.

The mode of calculation above outlined, and used in Experiments 1, 2, and 3, is illustrated by Fig. 1, by data from Experiment 3.

In Experiments 4, 5, and 6 the data directly determined have been the pH and the [CO_2] of the blood as drawn. From these the [$BHCO_3$] and p_{CO_2} have been calculated by the equations previously given.
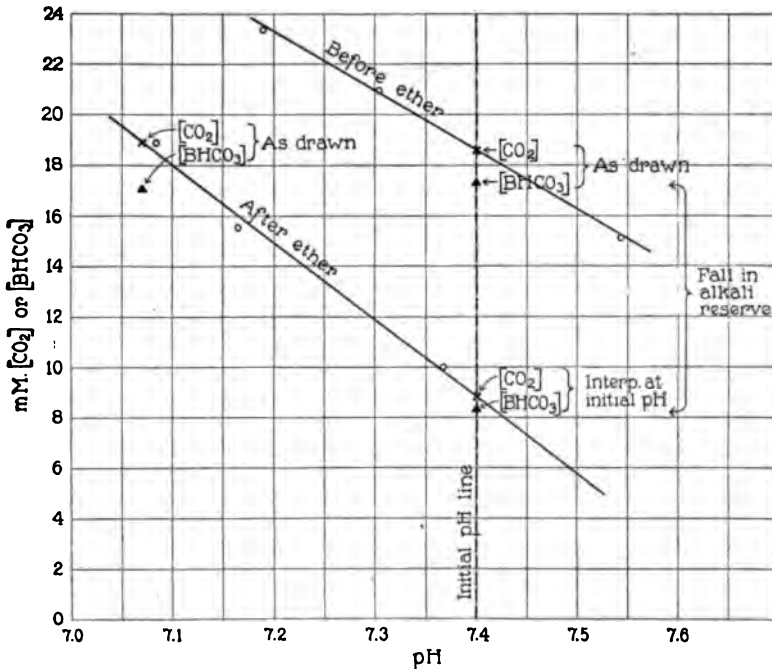
FIG. 1.

Extrapolation of the [$BHCO_3$] value of the bloods drawn after etherization to the values which those bloods would have under CO_2 tension such as would restore pH to its initial value, was necessary in order to measure the alkali reserve change at constant pH. For constructing the pH, [$BHCO_3$] lines necessary for the extrapolation in these experiments, there were available no graphs experimentally determined from equilibration data, since data on each blood were determined only at one point, with CO_2 tension as it existed when the blood was drawn. We have ac-
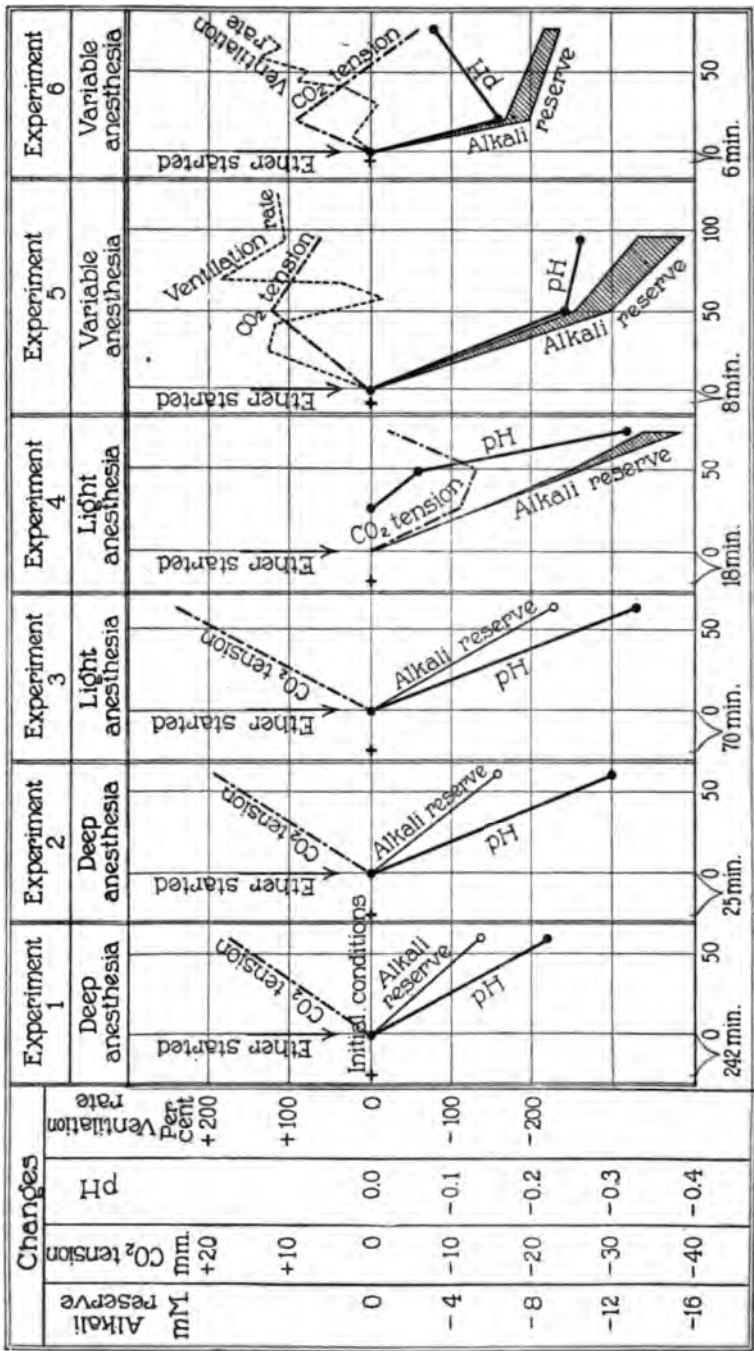
FIG. 2.

cordingly constructed two graphs in each case, using the two extreme limits of the $\dfrac{d[\text{BHCO}_3]}{d\text{pH}}$ slope as determined in a number of dog bloods from experiments of our own and from experiments in the literature. These limits are $\dfrac{d[\text{BHCO}_3]}{d\text{pH}} = -20$ to -28, [BHCO$_3$] being expressed in millimol units. The two sets of alkali reserve changes estimated by extrapolation with these two slopes are indicated, for Experiments 4, 5, and 6, in Fig. 2 by two lines, with a shaded area between them. The width of this area indicates the maximum error introduced by this method of extrapolation.

In Fig. 2 we have plotted the results as changes in alkaline reserve, CO$_2$ tension, pH, and ventilation rate from the initial values determined before anesthesia.

In our tables we have given the absolute values of our data.

Experiments and Results.

The details of the six experiments are shown in the protocols and tables and the results are given graphically in Fig. 2. In Experiments 1 and 2 the anesthesia was as deep as possible. In Experiments 3 and 4 it was as light as possible, the animal making spontaneous movements from time to time. In Experiments 5 and 6 the animals were given an uneven anesthesia, being at times deeply under and at times almost out of ether but with the anesthesia becoming more evenly settled at about the middle of the second stage as the experiment progressed.

In Experiments 1 to 4 no special precaution was taken to prevent fall in body temperature and as indicated in the protocol of Experiment 4 some cooling occurred. In Experiments 5 and 6 fall in temperature was prevented by covering the animal with a blanket throughout the experiment. The results, however, were substantially the same.

Ventilation rate was studied only in the last two experiments. There is continual fluctuation in the ventilation rate but in both of these experiments there is a tendency to its progressive increase in spite of the fact that in the latter part of the experiment the animals were held in the middle of the second stage of anesthesia.

SUMMARY.

In all the experiments it will be seen that the pH of the blood fell either at once or in a short time after the anesthesia was begun, and in the last three experiments it will be seen that although the pH remains low or continues to fall, the alkaline reserve also continues to fall.   In no case was any rise of pH above the initial value observed.   In all but one of the experiments the CO_2 tension was increased.

The evidence indicates that these changes do not occur as a compensatory mechanism to balance an acapnia.   A true acidosis occurs with increase of the hydrogen ion concentration of the blood and fall of the alkaline reserve, due either to introduction of acid into the blood or to withdrawal of base from it.

Experiment 1.

Young male. 10.12 a.m. first bleeding, left ventricle; 2.15 p.m. ether started, drop method, deep anesthesia; 3.15 p.m. second bleeding, left ventricle.

Blood Equilibrated at 38° (Defibrinated).

Sample.	Time drawn.	CO_2 tension.	Total $[CO_2]$.	pH	
				Calculated.	Rectified by graph.
		mm.	*mM.*		
1	Before ether.	18.7	10.9	7.465	7.465
		37.4	15.2	7.300	7.315
		56.2	18.8	7.206	7.193
		74.9	20.9	7.118	7.118
2	After 60 minutes deep etheriza-tion.	56.2	14.2	7.069	7.063*
		74.8	16.0	6.990	7.002*

* Graph drawn parallel to that of blood before ether.

Blood as Drawn.

Sam-ple.	Time drawn.	Determined.					Calculated.			
		Total [CO$_2$].	O$_2$ content.	O$_2$ capacity.	Total [CO$_2$] oxygenated at same pH.*	pH by interpolation of CO$_2$.	CO$_2$ tension.	[BHCO$_3$]		
								As drawn.	At initial pH.	
		mM.	*mM.*	*mM.*	*mM.*		*mm.*	*mM.*	*mM.*	
1	Before ether.	16.4	9.70	10.15	16.2	7.27	42	14.9	14.9	
2	After ether.	15.9	8.55	11.17	14.5	7.05	60	12.7	9.2†	

* $\dfrac{d\ CO_2}{d\ O_2} = 0.52$ at constant pH (unpublished data).

† From extrapolated CO$_2$ (see Fig. 1).

Experiment 2.

Young male (same as Experiment 1). 9.25 a.m. first bleeding, left ventricle; 9.49 a.m. ether started, drop method, deep anesthesia; 10.51 a.m. second bleeding, left ventricle.

True Plasma from Oxalated Blood Equilibrated at 38°.

Sample.	Time drawn.	CO$_2$ tension.	Total [CO$_2$].	pH	
				Calculated.	Rectified by graph.
		mm.	*mM.*		
1	Before ether.	40	18.2	7.224	7.224
		60	21.5	7.112	7.112
2	After 62 minutes deep ether.	20	9.8	7.257	7.257
		70	18.5	6.960	6.960

True Plasma (Oxalated) as Drawn.

Sample.	Time drawn.	Total [CO$_2$].	Calculated.			
			pH by interpolation of [CO$_2$].	CO$_2$ tension.	[BHCO$_3$]	
					As drawn.	At initial pH.
		mM.		*mm.*	*mM.*	*mM.*
1	Before ether.	18.2	7.22	40	16.9	16:9
2	After 62 minutes deep ether.	17.2	7.00	59	15.3	10.0*

* From interpolated [CO$_2$].

Experiment 3.

Young male (same as Experiments 1 and 2). 10.45 a.m. first bleeding, left
ventricle; 11.55 a.m. ether started, lightest possible anesthesia, drop
method; 12.25 to 12.37 p.m. breathing very violently; 12.37 to 1.00 p.m.
anesthesia very light, regular deep breathing, 33 per minute; 1.00 p.m.
second bleeding, left ventricle.

Oxalated Blood Equilibrated at 38°.

Sample.	Time drawn.	CO_2 tension.	Total $[CO_2]$.	pH	
				Calculated.	Rectified by graph.
		mm.	*mM.*		
1	Before ether.	21.8	15.1	7.545	7.550
		50.8	21.0	7.305	7.230
		72.5	23.4	7.190	7.195
2	After 65 minutes light ether.	21.4	10.0	7.367	7.360
		50.8	15.6	7.165	7.180
		72.5	18.9	7.086	7.070

Blood as Drawn (Oxalated).

Sample.	Time drawn.	Total $[CO_2]$.	pH by interpolation.	Calculated.		
				CO_2 tension.	$[BHCO_3]$	
					As drawn.	At initial pH
		mM.		*mm.*	*mM.*	*mM.*
1	Before ether.	18.6	7.40	41	17.7	17.
2	After 65 minutes light ether.	18.9	7.07	75	17.1	8.

* From $[CO_2]$ interpolated at pH 7.40.

Experiment 4.

. 1.10 p.m. first bleeding, left ventricle; 1.28 p.m. ether
method, light anesthesia; 1.55 p.m. second bleeding, right
y; 2.18 p.m. third bleeding, right femoral artery; 2.42 p.m.
ing, left femoral artery. Rectal temperature: initial and
9; final and minimum 37.3.

rue Serum as Drawn (Spontaneous Coagulation).

e drawn.	Determined.			Calculated.			
	[BHCO₃] titration.	Total [CO₂] gasometric.	Colorimetric pH.	CO₂ tension.	[BHCO₃] as drawn from gasometric CO₂.	[BHCO₃] at initial pH.	
						$\dfrac{d\,[\mathrm{BHCO_3}]}{d\,\mathrm{pH}} = -20$	$\dfrac{d\,[\mathrm{BHCO_3}]}{d\,\mathrm{pH}} = -28$ $\dfrac{d\,\mathrm{pH}}{d\mathrm{B}}$ observed.
	mM.	*mM.*		*mm.*	*mM.*	*mM.*	*mM.*
ether.	21.5	23.0	7.29	54	21.7	21.6	21.6
7 minutes ether.	12.8	16.8	7.29	40	15.9	16.0	16.0
) minutes ether.	12.8	13.8	7.23	37	12.9	11.7	11.2
4 minutes ether.	12.6	13.5	7.07	51	12.3	6.3	6.3

Experiment 5.

Young male. 10.02 a.m. first bleeding, left ventricle; 10.10 a.m. ether started, drop method; 10.27 a.m. cannula in trachea, anesthesia kept in middle of second stage; 11.00 a.m. second bleeding, left femoral artery; 11.47 a.m. third bleeding, right femoral artery. Rectal temperature: initial 38.2°; final 38.3°; maximum 38.6°; minimum 38.2°C.

Sample.	Time drawn.	Total [CO$_2$] oxalated blood as drawn.	Colorimetric pH. True serum as drawn.	Calculated for whole blood.			
				CO$_2$ tension.	[BHCO$_3$] as drawn.	[BHCO$_3$] at initial pH. $\frac{d[BHCO_3]}{d\,pH} = -20$	[BHCO$_3$] at initial pH. $\frac{d[BHCO_3]}{d\,pH} = -28$
		mM.		*mm.*		*mM.*	*mM.*
1	Before ether.	21.70	7.44	39	20.5	20.5	20.5
2	After 50 minutes varied ether.	16.78	7.20	47	15.4	10.6	8.7
3	After 97 minutes varied ether.	14.03	7.18	45	12.7	7.3	5.2

Time.	Ventilation rate.	Per cent Δ.
	liters per min.	
Day before.	5.6	
9.48 a.m.	3.9	
Mean before ether.	4.8	±0
10.35 a.m.	11.0	+129
10.52 "	10.4	+118
11.07 "	4.1	− 15
11.17 "	6.5	+ 35
11.20 "	13.6	+183
11.43 "	9.9	+106
12.14 p.m.	10.0	+108

Experiment 6.

About 4 year old male. 10.50 a.m. first bleeding, left ventricle; 10.56 a.m. ether started, drop method, kept about mid-second stage; 11.15 a.m. second bleeding, left femoral artery; 11.32 a.m. tracheal cannula introduced; 12.13 p.m. third bleeding, right femoral artery. Rectal temperature: initial 38.2°; final 38.3°; maximum 38.5°; minimum 38.2°C.

Separate Serum Equilibrated at 38°.

Sample.	Time drawn.	Atmospheric CO_2 tension.	Total $[CO_2]$.	Serum.			Calculated from colorimetric pH.			
				Electrometric pH 38°.	Colorimetric pH.		$[H_2CO_3]$	$[BHCO_3]$	$\log \frac{[BHCO_3]}{[H_2CO_3]}$	pK'
		mm.	*mM.*				*mM.*	*mM.*		
1	Before ether.	40.4	25.52	7.38	7.39		1.28	24.24	1.277	6.11
3	After 77 minutes varied ether.	39.4	18.65	7.19	7.19		1.25	17.40	1.144	6.05 Mean 6.08

True Serum as Drawn.

Sample.	Time drawn.	Total $[CO_2.]$	Colorimetric pH.	Calculated (using pK' = 6.08).			
				CO_2 tension.	$[BHCO_3]$ as drawn.	$[BHCO_3]$ at initial pH.	
						$\frac{d[BHCO_3]}{d pH} = 20$	$\frac{d[BHCO_3]}{d pH} = 28$
		mM.		*mm.*	*mM.*	*mM.*	*mM.*
1	Before ether.	25.32	7.36	40	24.06	24.1	24.1
2	After 19 minutes ether.	22.20	7.20	49	24.64	17.4	16.2
3	After 77 minutes.	18.16	7.28	34	17.08	15.5	14.8

Experiment 6—Concluded.

Time.	Ventilation rate.	Per cent Δ.
	liters per min.	.
Day before.	4.4	
10.30 a.m.	4.6	
Mean before ether.	4.5	± 0
11.05 a.m.	5.6	+ 22
11.24 "	4.1	− 9
11.35 "	5.8	+ 29
11.41 "	8.4	+ 87
11.47 "	7.9	+ 76
11.55 "	10.6	+136
12.02 p.m.	9.6	+113
12.08 "	10.2	+127

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STUDIES ON EXPERIMENTAL RICKETS.

XXI. AN EXPERIMENTAL DEMONSTRATION OF THE EXISTENCE OF A VITAMIN WHICH PROMOTES CALCIUM DEPOSITION.

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With the discovery in 1913 that certain fats contain a substance or substances which are essential for growth, this class of foodstuffs assumed an importance in nutrition which had not been hitherto accorded it. At first the evidence of the existence of the substance which subsequently became known as fat-soluble A, or vitamin A, rested upon the failure of experimental animals to grow when the fats carrying this substance were lacking in the food, and the resumption of growth when such fats were administered. Later xerophthalmia of a certain type was recognized as a pathological condition which invariably results from specific starvation for fat-soluble A. We have recently, however, convinced ourselves that a similar ophthalmia may be the result of disturbance of the balance of inorganic elements in the diet. Up to the present time no definite evidence has been brought forward to show whether one or more than one substance is contained in those fats which contain fat-soluble A which gives them their unique biological value (1). The great activity in several laboratories in the study of the cause or causes of rickets and related conditions has, during the last 3 years, brought to light the fact that there is a rôle played by fats in the etiology of this disease. This observation causes us to appreciate further the importance of the fat moiety of the food supply, and emphasizes the necessity

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of providing in the diet a regular and abundant supply of such fats as promote the normal development of the skeletal tissues.

Mellanby (2) was the first to associate the group of fats which, because of their content of fat-soluble A were frequently distinguished as "growth-promoting" fats with the prevention of rickets. His work focused attention upon the problem as to whether fat-soluble A is itself a substance essential for the normal growth of bone.

Mellanby (3) was so impressed with the power of butter fat to protect puppies against the abnormalities of bone growth that he stated:

"These facts of agreement from the point of view of physiological reaction seem to me strong evidence that the substance in fats stimulating the calcification of bone is the same as Fat-soluble A, i.e. the factor which stimulates growth in rats."

He further says:

"Rats as regards growth and puppies as regards rickets become more independent of the vitamin as they grow older. These facts are in favor of Fat-soluble A being also responsible for the anti-rachitic action of certain fats."

All of Mellanby's own data cannot be brought into line with this reasoning. For example, Dog 187 was fed bread, yeast, salt, orange juice, separated milk, and 10 cc. of cottonseed oil, but no meat. It developed rickets. Dog 305 had the same diet but with 10 gm. of meat. The bones of this animal are described as "practically normal" (3). Meat is essentially lacking in fat-soluble A. It does, however, contain phosphorus, and but little calcium, and serves to change the ratio between these elements in the diet. This, as has been pointed out, is of great importance in the etiology of rickets (4).

In his experiments Mellanby studied cod liver oil, butter fat, lard, suet, bacon fat, peanut oil, olive oil, coconut oil, linseed oil, babassu oil, rape seed oil, palm kernel oil, and hydrogenated fats. He states (3):

"Of the fats tested, cod-liver oil is the best. Suet and butter also have a potent influence on calcification. Lard is poor as compared with suet. Butter, heated and oxidized for four hours, loses some anti-rachitic action.

Cod liver oil similarly treated still has a strong anti-rachitic effect. The vegetable oils vary in their anti-rachitic action, the order of merit being somewhat as follows: pea-nut and coco-nut oils (best), rape-seed, cotton-seed, palm-kernel, olive, linseed, and babassu oils (worst). Hydrogenated fats are poor."

It will be seen from the foregoing quotations that the question as to whether fat-soluble A exerts an antirachitic effect, or whether such action is to be referred to some other principle which has in many cases at least, a similar distribution is still open.

In July, 1921 (5) we pointed out on the basis of experimental data that

"We have not found it possible to demonstrate experimentally a difference between the effects of cod liver oil and butter fat when the content of the diet in calcium and phosphorus is near the optimum, the other elements in the diet being satisfactory."

We stated further that

"These results suggest the possibility that a dietary essential distinct from the anti-ophthalmic substance (fat-soluble A) may exist. If this is the case this would appear to be present in butter fat in small amounts, but to be very abundant in cod liver oil."

Again, in January, 1922 (6) we set forth evidence which was all but conclusive that there is a specific calcium-depositing substance. We stated

"The results of this series of experiments were so consistent and decisive that we can deduce no other conclusion than that cod liver oil contains in abundance some substance which is present in butter fat in but very slight amounts, and which exerts a directive influence on bone development and enables animals to develop with an inadequate supply of calcium much better than they could otherwise do. This substance is apparently distinct from fat-soluble A, which is essential for growth and which is associated definitely with the prevention of ophthalmia (keratomalacia)."

Our own experience had convinced us that existing methods were incapable of differentiating beyond doubt between fat-soluble A and a special calcium-depositing substance should such exist. We therefore formulated a plan which involved a comparison of a selected list of fats in respect to three kinds of effects in nutrition. First, we tested cod liver oil, shark liver oil, butter fat, and several vegetable oils for potency in causing the cure of xerophthalmia

due to lack of fat-soluble A.   Secondly, we made comparative
tests of the same fats to determine their value in promoting growth
in young rats which were restricted to a diet so low in calcium
that satisfactory growth was not possible without the provision
of some substance which would make for a greater efficiency in
the utilization of calcium than that which could be effected in its
absence.   Thirdly, we further studied these same fats by means
of our "line test" to discover their relative values for inducing
the deposition of the line of calcium salts in rachitic bones.   With
the data which we have secured from these three distinct types of
tests, we are now in a position to interpret accurately the results
of much of the experimental data in the literature which is other-
wise confusing.

EXPERIMENTAL PROCEDURE.

I. Test for Fat-Soluble A.

The diet used for testing the value of different fats for the cure
of xerophthalmia had the following formula.

Lot 3392.

	per cent
Rolled oats	40.0
Casein	5.0
NaCl	1.0
$CaCO_3$	1.5
Dextrin	52.5

This diet is essentially lacking in fat-soluble A.   If 2 per cent of
butter fat or cod liver oil is included in place of an equivalent
amount of dextrin, xerophthalmia never develops, and the animals
are able to grow.

In making the tests referred to in this paper young rats of 40
to 60 gm. weight were restricted to Diet 3392 until the puffiness
of the eyelids was distinctly evident.   At this point the eyes were
frequently sealed shut while the rats slept, and were opened with
difficulty on awakening.   The malnutrition induced by this
diet progresses rapidly to a fatal termination unless a suitable
amount of fat-soluble A is provided at this stage.   On the addition
of sufficient fat containing fat-soluble A, at the time when the

edema of the eyelids is just becoming severe the swelling rapidly disappears, and the eyes return to a normal appearance within a few days. 2 per cent of cod liver oil, 3 per cent of shark liver oil, 3 per cent of burbot liver oil,[1] or 2 per cent of butter fat, were found to effect the prompt cure of incipient xerophthalmia under the conditions of our tests. Although we have data from other experiments which indicate that certain of the vegetable oils when fed liberally (8 to 20 per cent) from the beginning of the experiment tended to defer the onset of xerophthalmia, we have never found that such amounts of vegetable fats would cure the eye condition after it had once developed. It is, therefore, possible that there are traces of fat-soluble A in some vegetable fats. It should be specially noted here that 15 per cent of coconut oil did not cure or prevent xerophthalmia. We are convinced that a properly conducted *curative* test such as we have described is much more delicate than a *preventive* test can ever be made.

Hopkins (7) was the first to point out that oxidation destroys fat-soluble A. He showed that if oxygen is allowed to pass through heated butter fat the fat-soluble vitamin is readily destroyed. With this destruction the butter fat loses its power of inducing growth or of curing ophthalmia of dietary origin. Mellanby attempted (3) to make use of this means of destroying fat-soluble A in order to determine whether there is a distinct "anti-rachitic substance." He found butter fat of little value for protecting against rickets after it had been oxidized, whereas cod liver oil after the same treatment, *i.e.* heated to 120°C. for 4 hours while oxygen was passing through it, still protected his animals against rickets. He states:

"If it should happen that four hours' heating and oxidation at 120°C. also leaves a large amount of Fat-soluble A in the cod liver oil, it will go a long way, especially when considered together with the butter results, to clinch completely the identity of fat-soluble A and the anti-rachitic vitamine."

Mellanby used no method of testing for fat-soluble A as distinct from the calcium-depositing substance since he did not make use of the ophthalmia test for fat-soluble A.

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[1] We are indebted to Miss Ethel Kalmbach of Sturgeon Bay, Wis., who kindly furnished us a large sample of carefully prepared oil from the livers of the burbot.

We have found that cod liver oil treated with a stream of air bubbles at the temperature of boiling water for 12 to 20 hours no longer contains sufficient fat-soluble A to relieve rats from xerophthalmia when administered to the extent of 2 per cent of the diet. Cod liver oil which had been oxidized 4 hours, when fed as 2 per cent of the diet, cures xerophthalmia. Untreated cod liver oil under these conditions invariably causes complete recovery within 5 days. Likewise, 2 per cent of fresh butter fat, under exactly comparable experimental conditions, effects the disappearance of ophthalmia within 5 to 10 days. These results are sufficient to serve as a basis of comparison for our present purpose of the relative values of cod liver oil and butter fat for the cure of ophthalmia. The significance of these results in connection with the problem of the existence of a special calcium-depositing vitamin will be discussed later.

II. Tests of Fats for Their Protective Power against the Effects of Deficient Calcium Supply.

In a former paper (5) we have described experiments which showed clearly that a diet may be so deficient in calcium as to prevent growth in young rats. Butter fat failed to protect the animals from the effects of calcium deficiency, whereas the same diet supplemented with cod liver oil may promote good growth. We have since refined the technique of this type of experiment for studying the influence of fats on calcium metabolism. For this purpose we now use the following diet.

Diet 2947.

	per cent
Whole wheat	25.0
Whole maize	19.5
Rice polished	9.5
Rolled oats	9.5
Whole milk powder	5.0
Peas	9.5
Navy beans	9.5
Casein	10.0
NaCl	1.0
Dextrin	1.5

The dietary properties of this food mixture have been carefully determined.   The proteins are of very good quality and are abundant (about 23 per cent).   Its content of water-soluble B is entirely adequate.   It is somewhat deficient in fat-soluble A, but not sufficiently so as to induce xerophthalmia even if an animal were confined throughout life to it.   Its inorganic content was such as to induce skeletal deformities.   The diet was low in calcium, and not far from the optimum in phosphorus, and our problem was to see to what extent if any the different fats increase the efficiency of the animals in utilizing the small amount of calcium at their disposal.

This food mixture was fed with the following fats: cod liver oil 1.0 per cent; butter fat 10.0 per cent; shark liver oil 3.0 per cent; coconut oil 10.0 per cent; cottonseed oil 10.0 per cent; and olive oil 10.0 per cent.

An inspection of the charts shows that on this diet the animals cannot grow appreciably when the fat which is supplied them, is cottonseed or olive oil.   When cod liver oil, shark liver oil, or butter fat is fed, growth proceeds in a fairly satisfactory manner. Coconut oil is the only one of the vegetable oils examined which increased the efficiency of the animals in utilizing their very low calcium supply.   These data show clearly that there is a very remarkable property of certain fats which makes them of extraordinary importance in relation to calcium metabolism.

III. Tests of Fats for Their Power to Stimulate Healing in Rickets.

The data presented above relative to the values of several fats for relieving xerophthalmia, and for increasing the efficiency of the tissues in utilizing calcium when this element is present in very inadequate amounts in the diet, do not constitute a safe basis for deciding whether the effects in both types of experiments were due to fat-soluble A, or to two distinct substances.   The results of the experiments in which coconut oil was studied (Chart 4, Lot 3008) suggest strongly that this oil contains a substance which improves the utilization of calcium.   It has been pointed out above on the basis of experimental tests that coconut oil does not contain the substance, fat-soluble A, which

relieves xerophthalmia, since no improvement follows the ingestion of liberal amounts of this fat even in the incipient stages of the eye disease. Experiments directed toward preventing xerophthalmia by feeding coconut oil confirmed this view. We, therefore, supplemented the data obtained by the above described tests, with observations on the effects of the several fats on the initiation of the healing process in rickets. For this purpose we used the technique which we have called the "line test."

Diet No. 3143 employed in the "line test" consists of:

Diet 3143.

per cent

Whole wheat kernel	33.0
Whole maize kernel	33.0
Gelatin	15.0
Wheat gluten	15.0
NaCl	1.0
CaCO₃	3.0

The details concerning the properties of this diet and its effects on the bones have been given in another publication (8). It is only necessary to state here that it contains a very inadequate amount of fat-soluble A, but sufficient to prevent xerophthalmia during the interval necessary to develop an exaggerated rickets and to observe the incipient healing of the lesion. Although the diet contains an *excessive* amount of calcium, no deposition of calcium salts takes place.

A few words of explanation are necessary regarding the column in Table I headed "Number of days in preparatory period." In order to carry out the "line test" satisfactorily the animals are fed Diet 3143 until a pathological metaphysis has developed satisfactorily. This state we have come to recognize by certain peculiarities in the movements of the young rats which we use as the test animals. The gait is unsteady and the hind quarters waver from side to side. When they move off rapidly they hop, usually favoring one hind leg. We have found it safe to rely on young rats which exhibit this abnormality of movement to show the histological picture which is essential for the conduct of the test.

We have examined histologically the bones of a large number of rats which have been kept on this diet for varying periods, and

TABLE I.

Kind of oil.	Per cent.	Number of days in pre-paratory period.	Number of days fat was given.	Number of animals.	Results.
Cod liver oil.	2	21	5	6	Healing rickets.
" " "	2	25	6	2	" "
" " "	2	28	11	3	" "
" " "	2	49	5	3	" "
" " "	2	49	5	3	" "
" " "	0.2	70	5	1	Severe rickets, a few specks of calcium in cartilage.
" " "	0.4	70	5	1	Severe rickets, no healing.
" " "	0.6	70	5	1	Beginning healing of rickets.
Cod liver oil (oxidized 4 hrs.)	2	25	6	3	Healing rickets.
Cod liver oil (oxidized 12 hrs.)	2	28	11	3	" "
Cod liver oil (oxidized 20 hrs.)	2	29	10	3	" "
Butter fat.	30	21	14	2	Beginning healing.
" "	30	32	11	4	" "
" "	15	17*	12	4	3 of these animals showed occasional specks of calcium in cartilage.
" "	15	20	20	1†	Beginning healing rickets.
Shark liver oil.	2	34	11	3	Marked healing.
Burbot liver oil.	2	30	6	2	Healing well on.
" " "	2	30	10	4	" " "
Coconut oil.	20	24	15	5	Very slight evidences of healing.
Maize oil.	20	34	15	5	Severe rickets, no healing.
Olive "	20	34	15	5	" " " "
Cottonseed oil.	20	29	14	5	" " " "
Sesame oil.	20	24	15	5	" " " "

* This preparatory period was too short. The metaphyses were narrow and irregular. It was possible to mistake calcified matrix which had existed before the onset of the disease as evidence of healing.

† This animal was badly deformed and feeble after being fed the butter fat for 20 days. There was no clinical evidence that the butter fat had been at all beneficial.

have found considerable difference in the length of time required to prepare them for the test for the calcium-depositing vitamin.   We have gained the impression that it takes their bones a decidedly longer time in summer than in winter to deviate from the normal histology in the manner desired.   Certainly they do not all respond in a certain number of days so that it is not possible to state accurately when they will be ready.   We can, however, rely with certainty on the peculiarities of movement described above as a criterion of the time when a substance to be tested should be administered.   The age of the animals is an important factor in the rate at which they may be prepared for the test. Rats weighing 55 to 60 gm. are as a rule, in fall, spring, and winter, ready in 28 to 35 days.

We have studied in this way the fats already discussed, and have included maize oil and sesame oil among the vegetable oils examined.   Coconut oil was found to be distinctly more effective as a calcium-depositing agent than any of the other vegetables tested.   It was, however, inferior to butter fat or any of the fish oils tested.

The several oils studied are listed in Table I.   The amounts fed, the number of days of administration, the length of the preliminary period, the number of animals tested, and the results of the tests are given.   Since starvation causes the deposition of calcium salts in the bones under the conditions of this test, we have invariably kept accurate records of food consumption in making these tests.   The food consumption was adequate in all cases (9).

An inspection of Table I shows that cod liver oil, shark liver oil, and burbot liver oil, were highly effective in moderate doses in causing the deposition of calcium in the bones of rachitic animals.   Butter fat is also effective when fed in large amounts (15 to 30 per cent), but it was necessary to extend the time of administration to 14 days in order to obtain even a faint calcification of the bones.

Coconut oil, when fed at 20 per cent of the diet, caused in 15 days the deposition of small amounts of calcium salts in the bones under the conditions of our test.   Maize oil, olive oil, cottonseed oil, and sesame oil, were likewise fed at 20 per cent of the diet for 14 to 15 days, but in no case was there any tendency to the deposition of calcium salts.

Samples of cod liver oil which had been oxidized for 4, 12, and 20 hours, respectively, were tested for their calcium-depositing properties. Those oxidized 12 and 20 hours showed this potency in a degree apparently comparable with similar amounts of un-oxidized samples, notwithstanding the fact that they had entirely lost their power to cure xerophthalmia. The samples which had been oxidized for 4 hours still cured xerophthalmia when 2 per cent of the diet consisted of the oil. They likewise gave a positive test for calcium-depositing power.

DISCUSSION OF RESULTS.

We have shown experimentally that cod liver oil oxidized for 12 to 20 hours does not cure xerophthalmia in rats. It does, however, cause the deposition of calcium in the bones of young rats which are suffering from rickets. This shows that oxidation destroys fat-soluble A without destroying another substance which plays an important rôle in bone growth.

Coconut oil is shown to be lacking in fat-soluble A, since it will neither prevent nor cure xerophthalmia. This oil, on the other hand, contains a substance which stimulates the deposition of calcium salts in rickets in a manner similar to cod liver oil. It is, like butter fat, far less effective from a quantitative standpoint.

Cod liver oil, shark liver oil, and burbot liver oil, are highly effective for curing xerophthalmia, for protecting the body against the effects of a deficiency of calcium, and for the deposition of lime salts in rachitic bones.

Certain vegetable fats, among which are cottonseed oil, maize oil, sesame oil, and olive oil, do not possess the property of curing xerophthalmia, nor do they raise the efficiency of the tissues in utilizing calcium when there is an inadequate provision, nor of initiating healing in rickets.

Butter fat contains the calcium-depositing factor but in much smaller amounts than the fish oils we have examined. It is a much better source of fat-soluble A than of the substance which regulates calcium metabolism.

Our results are in harmony with those of Mellanby in that they show that coconut oil has an antirachitic effect. They prove conclusively, however, that this effect is not due to the presence

of fat-soluble A in this fat.   Mellanby did not appreciate the importance of calcium and phosphorus in his experimental diets as a factor in the causation of rickets.   Many of his apparently discordant results can, we believe, be accounted for on this basis rather than on the content of his diets in the calcium-depositing vitamin (3).

The question might be raised as to whether the effect of the substance which is designated fat-soluble A in protecting the functional activity of the eyes, is not one, and the calcium-depositing effect of certain oils which contain it, another physiological effect of one and the same substance.   A controversy involving this principle has been carried on for many years over the identity or non-identity of pepsin and rennin.   It may be postulated that the two properties which cod liver oil and certain other fats can be shown to possess, are referable to two side chains on the same molecule, and that in oxidation we have destroyed one and left the other intact.   It is, of course, not possible at present to prove or disprove either of these views.   The only evidence which bears on the question is the observation that coconut oil which had received no chemical treatment whatever, has been shown to possess demonstrable calcium-depositing properties, whereas it does not show a comparable antixerophthalmic effect.   This points to the two properties under discussion being due to distinct substances.

The evidence set forth in this paper demonstrates that the power of certain fats to initiate the healing of rickets depends on the presence in them of a substance which is distinct from fat-soluble A.   These experiments clearly demonstrate the existence of a fourth vitamin whose specific property, as far as we can tell at present, is to regulate the metabolism of the bones.

CHART 1. Lots 2765 and 3006 were fed diets which were essentially identical in their dietary properties except in so far as these were modified by the added fats.   Diet 2765 contained 1.0 per cent of cod liver oil and Diet 3006 contained 10.0 per cent of a bleached cottonseed oil (Wesson oil). The defects in these diets were limited, as far as we can definitely characterize them at present, to a deficiency in calcium which was closely comparable in the two diets, and in such organic factors as certain oils (*e.g.* cod liver oil or butter fat) can supply.

The difference in the well being of these two groups of rats, due entirely to the qualities of the fats which they were fed, was most remarkable and

CHART 1, LOT 2765

	Ration:
Wheat	25.0
Maize	20.0
Rice	9.5
Rolled oats	9.5
Peas	9.5
Navy beans	10.0
Casein	1.0
Whole milk powder	5.0
Cod liver oil	1.0

LOT 3006

	Ration:
Wheat	20.0
Maize	15.0
Rice	9.5
Rolled oats	9.5
Peas	10.0
Navy beans	10.0
Casein	10.0
NaCl	1.0
Whole milk powder	5.0
Cottonseed oil	10.0

GRAMS

2nd generation   < 4 >   Weeks

Lot 2765   Lot 3006

Y = birth of young

305

CHART 2, LOT 2766

Ration:	
Wheat	20.0
Maise	15.0
Rice	9.5
Rolled oats	9.5
Peas	10.0
Navy beans	10.0
Casein	10.0
NaCl	1.0
Whole milk powder	5.0
Butter fat	10.0

Y = birth of young

2nd generation

are illustrated by the curves in the chart. The group receiving the cod liver oil grew well and were fairly fertile and succeeded, notwithstanding the lack of calcium, in rearing most of their young. The group fed cottonseed oil, although they consumed about ten times as much fat, grew but little and failed early without having any young.

Lot 2765 received a suitable amount of both fat-soluble A and of the calcium-depositing vitamin, whereas Lot 3006 was essentially deprived of the calcium-depositing vitamin and secured but an inadequate amount of fat-soluble A in the seed products and milk powder which the diet contained. Lot 2765 was capable, because of the character of their diet, of utilizing effectively the small amount of calcium at their disposal, whereas Lot 3006 could not do this.

Chart 2. Lot 2766 had a diet like those described in Chart 1, but with 10 per cent of butter fat instead of cod liver oil or cottonseed oil. These animals grew fairly well but were decidedly inferior to those described in Chart 1, which received 1 per cent of cod liver oil. These records emphasize how very important it is to have in the diet certain fats possessing unique properties, when there is an unfavorable concentration of calcium in the food. Certain fats greatly protect the cells against their faulty chemical environment and enable them to utilize better than would otherwise be possible their very inadequate calcium supply. This, we are now in a position to assert, is due to the content in such fats of a special calcium-depositing vitamin which is often associated with fat-soluble A, but is distinct from it.

Chart 3. Lots 3012 and 3005 had the same food mixture discussed in Charts 1 and 2 except that the former had 3.0 per cent of shark liver oil and the latter 10 per cent of virgin olive oil. The contrast between the value of the fish oil on the one hand and the vegetable oil on the other in protecting the animals against the detrimental effects of a deficiency of calcium in the diet is very striking. Lot 3012 had a sufficient amount of both fat-soluble A and of the calcium-depositing vitamin. Lot 3005 had a suboptimal amount of the former, but very little of the latter, derived from the small amount of milk in its diet. The shark liver oil was not so effective as cod liver oil in raising the potential of the body cells so as to make them capable of utilizing an insufficient calcium supply, but it was distinctly better than butter fat for this purpose.

Chart 4. This chart is of special importance when its two groups of animals, Lots 3008 and 2947, are compared, and also these with Lot 3005, Chart 3, and Lot 3006, Chart 1.

Lot 2947 had the experimental diet previously discussed but without any added fats. On this they were able to grow but poorly, remaining undersized and infertile, and they deteriorated at an age when, on a better diet, they would still have been in possession of full vigor.

Lot 3008 is of special interest when compared with Lot 2947 which had the same diet without added fats and Lot 3005 (Chart 3) which had 10 per

CHART 2, LOT 2766

Ration:	
Wheat	20.0
Maize	16.0
Rice	9.5
Rolled oats	9.5
Peas	10.0
Navy beans	10.0
Casein	10.0
NaCl	1.0
Whole milk powder	5.0
Butter fat	10.0

Y = birth of young

are illustrated by the curves in the chart. The group receiving the cod liver oil grew well and were fairly fertile and succeeded, notwithstanding the lack of calcium, in rearing most of their young. The group fed cottonseed oil, although they consumed about ten times as much fat, grew but little and failed early without having any young.

Lot 2765 received a suitable amount of both fat-soluble A and of the calcium-depositing vitamin, whereas Lot 3006 was essentially deprived of the calcium-depositing vitamin and secured but an inadequate amount of fat-soluble A in the seed products and milk powder which the diet contained. Lot 2765 was capable, because of the character of their diet, of utilizing effectively the small amount of calcium at their disposal, whereas Lot 3006 could not do this.

CHART 2. Lot 2766 had a diet like those described in Chart 1, but with 10 per cent of butter fat instead of cod liver oil or cottonseed oil. These animals grew fairly well but were decidedly inferior to those described in Chart 1, which received 1 per cent of cod liver oil. These records emphasize how very important it is to have in the diet certain fats possessing unique properties, when there is an unfavorable concentration of calcium in the food. Certain fats greatly protect the cells against their faulty chemical environment and enable them to utilize better than would otherwise be possible their very inadequate calcium supply. This, we are now in a position to assert, is due to the content in such fats of a special calcium-depositing vitamin which is often associated with fat-soluble A, but is distinct from it.

CHART 3. Lots 3012 and 3005 had the same food mixture discussed in Charts 1 and 2 except that the former had 3.0 per cent of shark liver oil and the latter 10 per cent of virgin olive oil. The contrast between the value of the fish oil on the one hand and the vegetable oil on the other in protecting the animals against the detrimental effects of a deficiency of calcium in the diet is very striking. Lot 3012 had a sufficient amount of both fat-soluble A and of the calcium-depositing vitamin. Lot 3005 had a suboptimal amount of the former, but very little of the latter, derived from the small amount of milk in its diet. The shark liver oil was not so effective as cod liver oil in raising the potential of the body cells so as to make them capable of utilizing an insufficient calcium supply, but it was distinctly better than butter fat for this purpose.

CHART 4. This chart is of special importance when its two groups of animals, Lots 3008 and 2947, are compared, and also these with Lot 3005, Chart 3, and Lot 3006, Chart 1.

Lot 2947 had the experimental diet previously discussed but without any added fats. On this they were able to grow but poorly, remaining undersized and infertile, and they deteriorated at an age when, on a better diet, they would still have been in possession of full vigor.

Lot 3008 is of special interest when compared with Lot 2947 which had the same diet without added fats and Lot 3005 (Chart 3) which had 10 per

CHART 3, LOT 3012
Ration:

Wheat   20.0
Maize   22.0
Rice   9.5
Rolled oats   9.5
Peas   10.0
Navy beans   10.0
Casein   10.0
NaCl   1.0
Whole milk powder 5.0
Shark liver oil   3.0

LOT 3005
Ration:

Wheat   20.0
Maize   15.0
Rice   9.5
Rolled oats   9.5
Peas   10.0
Navy beans   10.0
Casein   10.0
NaCl   1.0
Whole milk powder 5.0
Olive oil   10.0

GRAMS
400
360
320
280
240
200
160
120
80
40

CHART 4, LOT 2008

Rations:	
Wheat	20.0
Maize	15.0
Rice	9.5
Rolled oats	9.5
Peas	10.0
Navy beans	10.0
Casein	10.0
NaCl	1.0
Whole milk powder	5.0
Coconut oil	10.0

LOT 2247

Rations:	
Wheat	25.0
Maize	12.5
Rice	9.5
Rolled oats	9.5
Peas	9.5
Navy beans	9.5
Casein	10.0
NaCl	1.0
Dextrin	1.5
Whole milk powder	5.0

GRAMS: 280, 240, 200, 160, 120, 80, 40

LOT 2008   LOT 2247   I = birth of young

CHART 5, Lot 2441

Ration:

Wheat	25.0
Maize	19.5
Rice	9.5
Rolled oats	9.5
Peas	9.5
Navy beans	9.5
Steak (round)	10.0
Whole milk powder	5.0
NaCl	1.0
$CaCO_3$	1.5

GRAMS

320 280 240 200 160 120 80 40

2nd generation   3rd generation   4th generation

cent of olive oil, and Lot 3006, Chart 1, which had 10 per cent of cotton-seed oil.

One striking feature about this series of curves is that Lot 3005 (Chart 3) was not quite so well nourished on its diet containing olive oil as was Lot 2947 which had the same diet with no fats added. This difference was not sufficiently pronounced to establish it as significant further than to show that olive oil certainly does not have the property of increasing the efficiency of the cells in functioning with an inadequate calcium supply.

The diet consumed by Lots 3005 and 2947 was the same as that of Lot 3006 (Chart 1) but modified in that cottonseed oil was included to the extent of 10 per cent. A comparison of these groups shows that the basal diet itself did not raise the efficiency of the cells in utilizing their insufficient calcium supply. Cottonseed oil and olive oil lack, therefore, a property which is possessed in high degree by cod liver oil, shark liver oil, burbot liver oil, and to a lesser extent, by butter fat. These fats of animal origin differ from the two plant oils mentioned in that the former exercise a pronounced protective action in directing the functioning of the anatomic elements of the osseous tissues, whereas the latter do not.

Of special interest is the record of Lot 3008, whose diet was the same as those just discussed except that it contained 10 per cent of coconut oil. With this diet there was an observable protective effect due to the coconut oil. There is, therefore, a slight difference between olive oil and cottonseed oil on the one hand, and coconut oil on the other, with respect to the special effect on the bone growth. The animals on this diet containing coconut oil were not so much protected by that oil as their growth curves would seem to indicate. They were very short bodied and stocky creatures with rough coats and presented rather a miserable appearance notwithstanding the fact that they grew at a slow rate to about two-thirds the normal adult size.

If we employ this diet without the coconut oil or other added fat and add 1.5 per cent of calcium carbonate, the animals thrive far better than they do with the calcium omitted and coconut oil included. See Chart 5.

It should be kept in mind in interpreting these records that the diets are not very faulty in any respect other than in a lack of calcium. The small content of milk powder containing some butter fat, and the small amount of fat-soluble A furnished by wheat and maize, gave the animals nearly enough of this principle to meet their minimal requirements. The butter fat contained in the milk powder supplemented the very feeble protective power of the coconut oil in respect to its effects on the bones due to the calcium-depositing vitamin. Any fat possessing in considerable degree the special property of increasing the efficiency of the cells in utilizing a low calcium supply would have exercised a much more marked beneficial effect on the general well being of the animals than did the coconut oil.

CHART 5. The records in this chart illustrate the fact that animals can undergo apparently normal development when furnished a very small amount of the calcium-depositing vitamin, provided the diet contains

somewhere near the optimal content of calcium and phosphorus. The adjustment of the calcium content of these diets at a very low level has enabled us to demonstrate the difference in the quality of fats in respect to their content of the calcium-depositing vitamin in a manner which would have been impossible if the diets had contained a more favorable concentration of calcium and phosphorus.

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ON A TYPE OF OPHTHALMIA CAUSED BY UNSATISFACTORY RELATIONS IN THE INORGANIC PORTION OF THE DIET. AN OPHTHALMIA NOT DUE TO STARVATION FOR FAT-SOLUBLE A, AND NOT CURABLE BY ITS ADMINISTRATION.

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All investigators appear now to be convinced that a deficiency of fat-soluble A in the diet will, if pronounced and the deprivation continued over a sufficient interval, cause the development of a type of ophthalmia. The ophthalmia induced in the rat by lack of fat-soluble A we know, from the studies of Mori in this laboratory, involves a xerosis of the conjunctivæ, and is accurately described as xerophthalmia.[1] This disease is now regarded as due to specific starvation for fat-soluble A, which is found abundantly in certain fats of animal origin. As pointed out by Osborne and Mendel, fat-soluble A is exceptionally abundant in cod liver oil.[2] The appearance of edema of the eyelids, and of a sticky exudate in the eyes, the development of corneal ulcers, and the appearance of hypopyon, followed by a xerosis of the conjunctivæ, are now generally regarded as evidence of the absence or paucity of fat-soluble A in the food supply.

During the last few years we have occasionally observed cases of "sore eyes" in rats which were provided with an abundance of fat-soluble A. We were at a loss to explain this occurrence except through possible infection of the eyes by bacteria. We felt that if this condition had been due to infection resulting from the

[1] Mori, S., The primary changes in the eyes of rats which result from deficiency of fat-soluble A in the diet, *J. Am. Med. Assn.*, 1922, lxxix, 197.

[2] Osborne, T. B., and Mendel, L. B., The influence of cod liver oil and some other fats on growth, *J. Biol. Chem.*, 1914, xvii, 401.

breaking down of the natural barriers of defense it should have occurred among our enfeebled animals irrespective of the nature of the defects in their diets which caused their enfeeblement. This has not been the case. It has been confined to experimental

TABLE I.

Percentage Composition of Diets.

Ration No.	Wheat germ.	Wheat gluten.	Egg albumin.	Gelatin.	Casein.	Dextrin.	Agar-agar.	Butter fat.	Salt mixture. No.	Salt mixture. Amount.	CaCO₃	NaCl	KH₂PO₄	NaH₂PO₄	KCl	MgO
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.		gm.	gm.	gm.	gm.	gm.	gm.	gm.
3407	3*	15†		15		56.96‡	2	2	XXI	3.9	1.5			0.64		
3408	3*	5†		5	5.0	72.6‡	2	2	XXI	3.9	1.5					
3369	3	12	10	10		52.2	2	5	XX	4.1			1.7			
3399	3				18.0	68.0	2	5	185	3.7						0.3
3400	3	12	10	10		52.4	2	5	XXI	3.9			1.7			
3401	3*	12†	10§	10		54.3‡	2	2	XXI	3.9	1.5			1.3		
3402	3				25.0	59.4	2	5	XXI	3.9			1.7			
3417	3*				31.1	59.4‡	0	2	None.		3.0	1.0			0.5	
3370	3*				25.0	59.2	2	5	XX	4.1			1.7			
3366	3*	12†	10§	*10		56.2‡	0	2	XX	4.1	1.5		1.2			
2074	3*					16.8‖	0	3	IX	5.2						
3015	4				18.0	65.2	2	10	XII	0.8						
3369-A	3	12	10	10		54.3	2	5	185	3.7						
3402-A	3				25.0	59.6	2	5	185	3.7			1.7			
3305-A	4				18.0	69.3	2	5	185	3.7						
3418	3*				29.7	62.86‡	0	2	None.		0.093	1.0		0.85		0.5
3442	3*				20.0	70.5‡	0	2	"		3.0	1.0			0.5	
2946	4				18.0	59.3	2	10	185	3.7	3.0					
3003	4				18.0	63.9	2	10	XI	2.1						

* Wheat germ extracted with ether and then with chloroform.

† Wheat gluten purified. See text.

‡ Dextrin made from specially purified starch.

§ Egg albumin purified. See text.

‖ This diet contained: polished rice, 75.0 gm.; Salt Mixture IX, 5.2 gm.; butter fat, 3.0 gm.; dextrin, 16.8 gm. The latter carried the alcoholic extract of 10 gm. of wheat germ.

groups employed in studying the effects of salt mixtures differing widely in composition. It may develop in animals which are growing fairly rapidly and present a moderately well nourished appearance. As soon as the eye disease appears the rats, as a rule, fail rapidly unless a suitable change is made in the diet.

TABLE II.

Composition of Salt Mixtures in Terms of Salts.

No. of salt mixture.	NaCl	MgSO₄, anhydrous.	NaH₂PO₄ + H₂O	K₂HPO₄	CaH₄(PO₄)₂ + H₂O	Ferric lactate.	Calcium lactate (5H₂O)	KCl	K₂CO₃	CaHPO₄	CaCO₃	MgO	NaHCO₃	FeSO₄ + 7H₂O
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
185	0.173	0.266	0.347	0.954	0.540	0.118	1.300							
IX	2.000							0.500		1.500	0.500	0.500		0.200
XX	0.500							1.000			1.500	0.200	0.700	0.200
XII	0.068							0.155	0.481		0.025	0.058		0.200
XXI	0.500							1.000			1.500		0.700	
XIII	0.246							0.193	0.835		0.177	0.122		
XI	0.319	0.266		1.429		0.111								

TABLE III.

Composition of Salt Mixtures in Terms of Elements.

No. of salt mixture.	Percentage of salts in diet.	K	Na	Ca	Mg	S	Cl	P	Fe	Employed in Diet No.
	per cent	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	
185	3.7	0.4280	0.1258	0.2742	0.0532	0.1003	0.1049	0.3803	0.0229	3399
IX	5.2	0.262	0.7870	0.5490	0.300	0.023	1.4400	0.2700	0.040	2074
XX	4.1	0.524	0.3890	0.600	0.120	0.023	0.7790	0.0000	0.040	3369
XII	0.8	0.3530	0.0660	0.010	0.035	0.137	0.115	0.153	0.000	3015
XXI	3.9	0.524	0.3890	0.600	0.000	0.023	0.7790	0.0000	0.040	3407
XIII	1.6	0.564	0.097	0.071	0.073	0.137	0.2410	0.153	0.000	3018
XI	2.1	0.253	0.126	0.000	0.053	0.071	0.194	0.641	0.023	3003

TABLE IV.

Inorganic Elements in Entire Rations per 100 Gm.

Ration No.	K	Na	Ca	Mg	S	Cl	P	Remarks.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	
3407	0.533	0.517	1.202	0.010	0.173	0.781	0.217	Sore eyes.
3408	0.533	0.411	0.202	0.010	0.121	0.781	0.088	" "
3369	1.142	0.523	0.620	0.143	0.167	0.920	0.463	Very sore eyes.
3399	0.428	0.126	0.274	0.233	0.237	0.105	0.533	Eyes normal.
3400	1.142	0.522	0.620	0.023	0.167	0.920	0.463	Sore eyes.
3401	0.533	0.623	1.202	0.010	0.153	0.781	0.359	" "
3402	1.021	0.411	0.602	0.010	0.223	0.781	0.633	" "
3417	0.271	0.416	1.202	0.010	0.246	0.846	0.298	" "
3370	1.021	0.411	0.602	0.130	0.223	0.781	0.633	" "
3366	0.877	0.411	0.602	0.730	0.153	0.781	0.345	" "
2074	0.289	0.809	0.555	0.319	0.076	1.477	0.340	" "
3015	0.353	0.066	0.010	0.035	0.137	0.155	0.153	Eyes normal.
3369-A	0.559	0.259	0.294	0.076	0.244	0.246	0.456	Recovery from sore eyes.
3402-A	0.925	0.148	0.276	0.063	0.300	0.107	1.013	Recovery from sore eyes.
3305-A	0.440	0.155	0.277	0.066	0.250	0.108	0.576	Eyes remain normal. Rats recover from ophthalmia on this diet.
3418	0.271	0.558	0.039	0.010	0.236	0.846	0.477	Sore eyes.
3442	0.271	0.416	1.202	0.010	0.162	0.846	0.203	" "
2946	0.440	0.155	1.477	0.066	0.250	0.108	0.576	Eyes normal.
3003	0.265	0.155	0.003	0.066	0.084	0.197	0.837	" "

From a study of our data involving the observation of rats on numerous types of diets we have come to the conclusion that there is a second type of ophthalmia produced by faulty diet. This condition may or may not be identical in its finer details with that which results from vitamin A starvation. The second type of

TABLE V.

Showing Types of Salt Supply Which Induce Ophthalmia and Others on Which Recovery Occurs.

Ration No.	K	Na	Ca	Mg	S	Cl	P	Remarks.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
3369	1.142	0.523	0.620	0.143	0.167	0.920	0.463	Eyes very sore in 60 to 75 days.
3369-A	0.559	0.259	0.294	0.076	0.244	0.246	0.456	Animals which developed ophthalmia on Diet 3369 recovered promptly on Diet 3369-A.
3402	1.021	0.411	0.602	0.010	0.223	0.781	0.633	Eyes sore in 60 to 70 days.
3402-A	0.925	0.148	0.276	0.063	0.300	0.107	1.013	Animals which developed ophthalmia on Diet 3402, recover on Diet 3402-A.
3305-A	0.440	0.155	0.277	0.066	0.250	0.108	0.576	Recovery takes place on this diet.
3399	0.428	0.126	0.274	0.233	0.237	0.105	0.533	Notwithstanding the high magnesium content of this diet the eyes remain normal.*
2946	0.440	0.155	1.477	0.066	0.250	0.108	0.576	Eyes normal, notwithstanding the high calcium content.

* In our studies of the nutritive properties of certain plant seeds, we have added as much as 1 per cent of magnesium oxide to the diet without any eye symptoms or other pronounced ill effects appearing over periods covering 7 to 8 months.

ophthalmia we believe to be caused by an unfavorable relationship between certain inorganic elements in the food of the animals. As far as one can tell by an inspection of the external appearance of the eyes of rats which are suffering from the ophthalmia due

to an excessive or an unfavorable supply of mineral elements, the condition is indistinguishable from the ophthalmia resulting from lack of fat-soluble A.

Although we have accumulated experimental data which are very convincing, we do not regard the etiology of this form of ophthalmia as completely elucidated. Considerable time must elapse before the completion of our study of this problem. Since there is now great activity in a number of laboratories in the study of the distribution and properties of fat-soluble A, we report our more significant results because of the danger that confusion may result from the feeding of diets containing even liberal amounts of fat-soluble A, but otherwise so constituted as to lead to the development of the ophthalmia which we are now discussing, which has no connection with vitamin A deficiency.

<center>DISCUSSION OF TABLES.</center>

In Table IV the compositions of nineteen diets are shown. Some of these, as will be seen in the column under "Remarks," induced ophthalmia, whereas others did not. Certain of the diets were composed of highly purified food substances, whereas some contained wheat gluten and egg albumin which had not been treated so as to remove their non-protein constituents. The salt mixtures employed in these diets differed widely in composition. All diets contained fat-soluble A, the amount varying from the minimum on which good growth can be obtained to very liberal quantities of this vitamin. The butter fat was found in control experiments to contain apparently the usual content of fat-soluble A.

In Table II the compositions of the salt mixtures employed are given in amounts of the salts used in their preparation.

In Table III the compositions of the salt mixtures are given in terms of their content of the elements of physiological significance.

Table IV shows the amounts of mineral elements in certain diets on which rats develop ophthalmia notwithstanding the presence of such amounts of fats carrying fat-soluble A as will not only prevent xerophthalmia of the vitamin deficiency type, but will promote growth and well being over a long period. The salt composition of certain diets is shown on which the eyes of animals remain indefinitely in a normal condition. Others are

tabulated on which ophthalmia developed, and still others to which the rats were transferred, which led to prompt recovery.

Whenever the new type of ophthalmia developed the animals were receiving what may be regarded as somewhat excessive amounts of total mineral nutrients.[3] As will be seen from Table IV the one element which was constantly present in high concentra- ·tion was chlorine. We have not yet tested diets which fulfill this condition, but which were low in their sodium content. We may contrast on the one hand Lots 3407, 3408, 3369, 3400, 3401, 3402, 3417, 3370, 3366, 2074, 3418, and 3442, all of which developed the eye condition, with Lots 3399, 3015, 3369-A, 3402-A, 3305-A, 2946, and 3003, which did not. At first thought those which received Salt Mixture 185 (Lots 3399, 3369-A, 3402-A, 3305-A, and 2946) may appear to form exceptions to this statement. An inspection of Table II will show, however, that the 3.7 gm. of this salt mixture which was contained in each 100 gm. of food, contained 1.30 gm. of calcium lactate, a salt containing 5 mole- cules of water and but 14.48 per cent of calcium. If the calcium in this mixture had been added in the form of carbonate the total amount of the salt mixture in 100 gm. of the food would have amounted to but 2.87 per cent of the diet. Salt Mixture 185 does not induce ophthalmia even when fed supplemented with 3.0 per cent of calcium carbonate (Diet 2946), or with potassium phosphate 1.7 per cent (Diet 3402-A), or with magnesium oxide 0.3 per cent (Diet 3399). These experiments suggest, therefore, that excessive calcium, potassium, or magnesium is not the cause of this ophthalmia.

The most significant data presented in the tables are brought together in Table V. Diet 3369 was very high in potassium, and contained somewhat over what we have regarded as a satisfactory amount of sodium, and about the optimal calcium and phosphorus contents, but was very high in chlorine. This diet has in our experience invariably induced ophthalmia in 60 to 75 days. The same may be said regarding Diet 3402.

[3] When young rats are restricted to a salt-free diet they also develop an ophthalmia which is apparently similar to that described here as the result of excessive chlorine ingestion (Na ?). The phenomenon is, there- fore, not entirely specific, but involves certain disturbances in the content of certain inorganic ions in the body fluids. This problem is receiving further study.

Animals which had developed the eye disease on Diet 3369 recovered promptly when Salt Mixture XX, together with 1.7 per cent of KH_2PO_4 was replaced by 3.7 per cent of Salt Mixture 185. This change reduced the content of the diet in all elements other than sulfur, which was increased somewhat, and phosphorus, which remained essentially unchanged. Animals fed Diet 3402 developed ophthalmia and recovered promptly when changed to Diet 3402-A, which differed only in the salt mixture added. The cure resulted, although the potassium content of the diet was nearly doubled. The sodium, calcium, and chlorine were reduced significantly.

Rats which had developed ophthalmia were also cured by transferring them to Diet 3305-A, which contained only 3.7 per cent of Salt Mixture 185. In this case we brought about a general reduction of mineral elements in the diet.

Diet 3399 contained 3.7 per cent of Salt Mixture 185, together with 0.3 per cent of magnesium oxide. The eyes remained entirely normal. In other experiments we have fed 1.0 per cent of magnesium oxide without causing ophthalmia.[4]

Diet 2946 contained 3.7 per cent of Salt Mixture 185, together with 3.0 per cent of calcium carbonate. The diet contained an excessive amount of calcium. The eyes of the animals on this diet remained normal.

We have repeatedly observed that rats fed diets which are optimal in composition with respect to all factors other than fat-soluble A, which is either lacking or nearly so, do not all develop xerophthalmia in the same length of time. It has been our experience that a deficiency of fat-soluble A does not cause xerophthalmia so quickly when the diet is optimal in respect to all other factors as it does when one or another factor other than vitamin A is of poor quality. In certain cases we have found the protein in the diet to exert a protective action in this respect. It may, therefore, be found in further work that a similar protective action will be exerted by other dietary factors in connection with the salt type of ophthalmia.

CONCLUSIONS.

From the data which we have discussed it is evident that the one constant factor which operated in the experiments in which

[4] McCollum, E. V., and Simmonds, N., Unpublished data.

ophthalmia was induced was a high content of chlorine. It is possible, however, that a high sodium content in the diet may contribute to cause this pathological condition. We suggest provisionally, therefore, that these are the etiological factors involved in inducing an eye condition which may be easily confused with the xerophthalmia due to lack of fat-soluble A. It may, of course, be found that the provision of excessive amounts of certain other inorganic elements may intensify the effect of chlorine, or sodium, or both, in hastening the onset of the eye disease. The whole question of the interrelation of salt effects in mammalian nutrition is still little understood.

Method of Purification of Egg Albumin.

1 pound of commercial powdered egg albumin (Merck) was placed in 10 liters of distilled water, and stirred occasionally until solution was complete. It was then heated over asbestos, and when the temperature had reached 80°C. acetic acid was added in amount sufficient to cause complete coagulation. After standing until the solution had cooled, the water was drained off on cheese-cloth, the coagulum returned to the vessel, and again brought to a boil with water acidulated with acetic acid. This process was repeated four times. Finally the albumin was drained on cheese-cloth, and dried in a current of warm air. It was then ground to a powder. This treatment is sufficient to remove practically all of the phosphorus, chlorine, and all the basic elements, from egg albumin.

Wheat Gluten.

500 gm. of commercial wheat gluten were treated with 5 liters of water to which acetic acid had been added to make a solution 0.2 of 1 per cent. The gluten was kept in the water and stirred at frequent intervals. At the end of the laboratory day the gluten was allowed to settle, the supernatant liquid removed, and replaced with a fresh solution. The treatment was continued during 6 days. The gluten was finally strained off on muslin and dried in a current of air. Wheat gluten treated in this way is almost free from phosphorus.

Purification of Starch.

5 pounds of commercial corn-starch were treated with 7 liters of 0.1 per cent hydrochloric acid. The starch was stirred at frequent intervals during the working day. The washing of the starch with 0.1 per cent hydrochloric acid was continued during 6 days. The starch was then washed with water, and the wet material acidified with citric acid and placed in a porcelain dish in an autoclave and heated at 18 pounds pressure for 1 hour to dextrinize it. The dextrin was then dried with a current of air.

CREATININE AND CREATINE IN MUSCLE EXTRACTS.

III. CONCERNING THE PRESENCE OF ENZYMES IN MUSCLE TISSUE WHICH HAVE CREATINE AND CREATININE AS THEIR SUBSTRATES.

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The determination of the presence or absence in muscle tissue of enzymes which affect the formation or destruction of creatine and creatinine and the transformation of creatine to creatinine is obviously of importance in connection with the problem of urinary creatinine.

Gottlieb and Stangassinger (1) reported results which they considered justified the belief of the presence of such enzymes in muscle tissue. Their findings were confirmed by other workers. Yet the negative results of Voegtlin and Towles (2) and others and the fact that the earlier methods of analysis were unreliable, seemed to justify a reexamination of the question.

Both Myers and Fine (3) and Hammett (4), using more exact methods of analysis, observed that when muscle extracts are aseptically incubated at body temperature there takes place in them an increase in creatinine at the expense of the creatine. This would seem to show that the process is enzymatic, were it not for the fact that the same change occurs in pure solutions of creatine in water, although at a slower rate.

In the experiments to be recorded in this paper the extracts which were used were made from the striated muscles of mature albino rats of both sexes. The muscle tissue was ground up in a meat chopper, macerated with fine sand and an equal weight of Tyrode's solution, and 10 cc. of toluene were added. The juice was then squeezed from the mixture by a filter press. The resultant extract was then diluted with an equal volume of Tyrode's solution, unless otherwise noted, and 5 cc. portions were

TABLE I.

Effect of Dialysis on the Formation of Creatinine from Creatine in Muscle Extracts When Incubated at 38°C.

Series		Fresh extract.	Un-dialyzed extract.	After 24 hours incubation and dialysis.				After 24 hours incubation when dialyzed extract and dialysate are separated.		
				Dialyzed extract.	B/A	Dialysate.	C/B	Dialyzed extract.	Dialysate.	C'/B'
			A	B		C		B'	C'	
		mg	*mg*	*mg*	*per cent*	*mg*	*per cent*	*mg*	*mg*	*per cent*
I	Creatinine.	0.070	0.171	0.172	0.4	0.178	3.5			
	Total creatinine.	5.97	5.76	5.77	0.2	5.90	2.3			
II	Creatinine.	0.078	0.145	0.145	0.0	0.148	2.1	0.263	0.229	−12.9
	Total creatinine.	5.48	5.48	5.55	1.3	5.55	0.0	5.48	5.51	0.5
III	Creatinine.	0.083	0.175	0.174	− 0.6	0.174	0.0	0.314	0.304	− 3.2
	Total creatinine.	5.23	5.19	5.20	0.2	5.23	0.6	5.21	5.20	0.2
IV	Creatinine.	0.056	0.144	0.141	−2.1	0.154	9.2	0.216	0.168	−22.2
	Total creatinine	4.19	4.22	3.31	−21.6	5.04	52.3	3.28	5.11	55.8
V	Creatinine.	0.069	0.186	0.185	− 0.5	0.186	0.5	0.355	0.292	−17.7
	Total creatinine.	5.95	6.02	6.05	0.5			5.98	6.04	1.0
VI	Creatinine.			0.216		0.205	−5.1	0.513	0.358	−30.6
	Total creatinine.			6.76		6.72	−0.6	6.00	5.95	− 0.8

VII	Creatinine.	0.182		0.182	0.0	0.400	0.312	−22.0
	Total creatinine.	6.69		6.72	0.4	5.96	6.00	0.7
VIII	Creatinine.	0.148		0.147	−0.7	0.229	0.213	− 7.0
	Total creatinine	4.79		4.79	0.0	4.80	4.80	0.0
IX	Creatinine.	0.154		0.154	0.0	0.366	0.352	− 3.8
	Total creatinine.	5.68		5.71	0.5			
X	Creatinine.	0.200		0.212	6.0	0.388	0.372	− 4.1
	Total creatinine.	5.75		5.80	0.9	5.80	5.73	− 1.2

invariably used for the analyses.   The methods of analysis were
the same as those previously described (4, 5).   The temperature
of the incubator was maintained at 38°.   The extracts were pro—
tected from microorganisms by toluene.   All the reported values
for creatinine and total creatinine are averages of duplicate deter—
minations.   Those for creatinine represent the so called "pre-
formed creatinine;" those for total creatinine, the preformed
creatinine plus the creatine as creatinine.

An attempt was first made to separate the accelerating agent
from the muscle extracts by dialysis.   Samples of extract were
dialyzed for 24 hours in the incubator, either in parchment or
collodion thimbles, against an equal volume of Tyrode's solution.
An equal amount of phosphate buffer mixture was added to
each.   This gave a pH between 6.9 and 7.0, determined color-i
metrically.   After the preliminary period of dialysis, the dialy-
sate and the dialyzed extract were separated and aliquots incu-
bated for a second 24 hours.   Table I gives the results of the
analyses made at the various periods.

It is seen that the amount of creatinine formed in the extract
is the same whether the extract is diluted 1:1 before incubation
or whether it is dialyzed against an equal volume of the diluent
during incubation.   The almost uniform appearance of equal
concentrations of creatinine and total creatinine in the dialyzed
extract and the dialysate after 24 hours incubation shows the
easy diffusibility of these compounds.   The results of the second
24 hour period of incubation are therefore strictly comparable.

It appears from this series of experiments as if the muscle ex-
tract as such provides a more favorable milieu for the transforma-
tion of creatine to creatinine than does its dialysate.   But since
the creatinine increase in the former is only 13.7 per cent greater
than in the latter, the evidence for an enzyme participation is
rather weak; unless it be believed that the enzyme, if present, is
dialyzable.

Since no change in total creatinine occurred in these experi-
ments or those to follow, it is evident that the increase in creatinine
is at the expense of the creatine.

These differences in creatinine might be due to differences in
hydrion concentration between extract and dialysate which Loeb
(6) has shown to exist in similar experiments, and from the fact

that I have shown such differences result in differences in rate of creatinine formation (4).

During experiments which were made in the study of the course of the reaction, creatine-creatinine, it was noted that the degree of dispersion of the material in the colloid state[1] decreased with time. Advantage was taken of this fact to effect a partial separation of the colloids from the extract by centrifugation after 4 hours incubation. The centrifuged extracts were then incubated further, and the rate of creatinine formation in them compared with uncentrifuged extracts. The results of this series are given in Table II. The values indicate that the partial removal of colloids has slightly retarded creatinine formation. This confirms the findings of the dialysis experiments. The degree of difference is too slight to allow the conclusion that enzyme has been pulled out of the extract by adsorption.

Attempts were next made to destroy any enzyme which might be present by NaF, HgCl₂, and KCN. The first of these did not influence the reaction at all. The others interfered with the colorimetric procedure and were consequently useless.

Studies were now made of the rate of creatinine formation. 5 cc. lots of muscle extract were placed in test-tubes and put in the incubator. Creatinine and total creatinine were determined in the fresh extract and the incubated samples at 2 hour intervals for the ensuing 24 to 30 hours. The results of two such series are plotted on Chart 1. They show that the rate of creatinine formation is more or less periodic. Phases of acceleration, simulating the course of an autocatalyzed reaction are abruptly cut short, and then the process is repeated. Similar periodic changes in rate of reaction have been observed by Bredig and Weinmayr (7) and Bray (8).

It was noticed during the first of these experiments that marked changes were taking place in the degree of dispersion of the colloids in the muscle extracts. There is first a gel stage, and then flocculation sets in. These changes occur gradually. Similar changes in colloid equilibrium with time have been noted by du Noüy (9) in blood serum and by Davis, Oakes, and Browne (10)

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[1] From now on the term "colloids" will be used for the sake of brevity instead of "materials in the colloid state."

TABLE II.

Effect of Partial Removal of Colloid Material from Muscle Extracts by Centrifugation on the Formation of Creatinine from Creatine during Incubation at 38°C.

Series	I		II		III		IV	
		A/B		A/B		A/B		A/B
	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent
Initial creatinine.	0.201		0.059		0.074		0.067	
Creatinine after 2 hrs. Untreated extract A			0.103		0.130		0.107	
Centrifuged extract B.......			0.094	9.6	0.117	11.1	0.095	12.6
Total creatinine, initial......	4.89		4.50		6.13		4.78	
Total creatinine, 2 hrs. Untreated extract A.........			4.47		6.03		4.76	
Centrifuged extract B.......			4.47	0.0	6.13	−1.6	4.78	−0.
Creatinine after 24 hrs. Untreated extract A........	0.304		0.187		0.306		0.218	
Centrifuged extract B.......	0.295	3.1	0.176	6.3	0.295	3.7	0.193	13
Total creatinine, 24 hrs. Untreated extract A	4.91		4.50		6.03		4.76	
Centrifuged extract B.......	4.96	−1.0	4.47	−0.7	6.08	−0.8		
Creatinine after 48 hrs. Untreated extract A.........	0.411		0.374				0.331	
Centrifuged extract B.......	0.408	0.7	0.338	10.7			0.336	−1
Total creatinine, 48 hrs. Untreated extract A.........	4.89		4.47				4.76	
Centrifuged extract B.......	4.91	−0.4	4.47	0.0			4.76	.0
Creatinine after 72 hrs. Untreated extract A.........			0.515				0.485	
Centrifuged extract B.......			0.490	5.1			0.484	.2
Total creatinine, 72 hrs. Untreated extract A.........			4.47				4.80	
Centrifuged extract B.......			4.50	0.7				
Creatinine after 100 hrs. Untreated extract A.........			0.617		·			
Centrifuged extract B.......			0.620	−0.5				
Total creatinine, 100 hrs. Untreated extract A.........			4.47					
Centrifuged extract B.......								
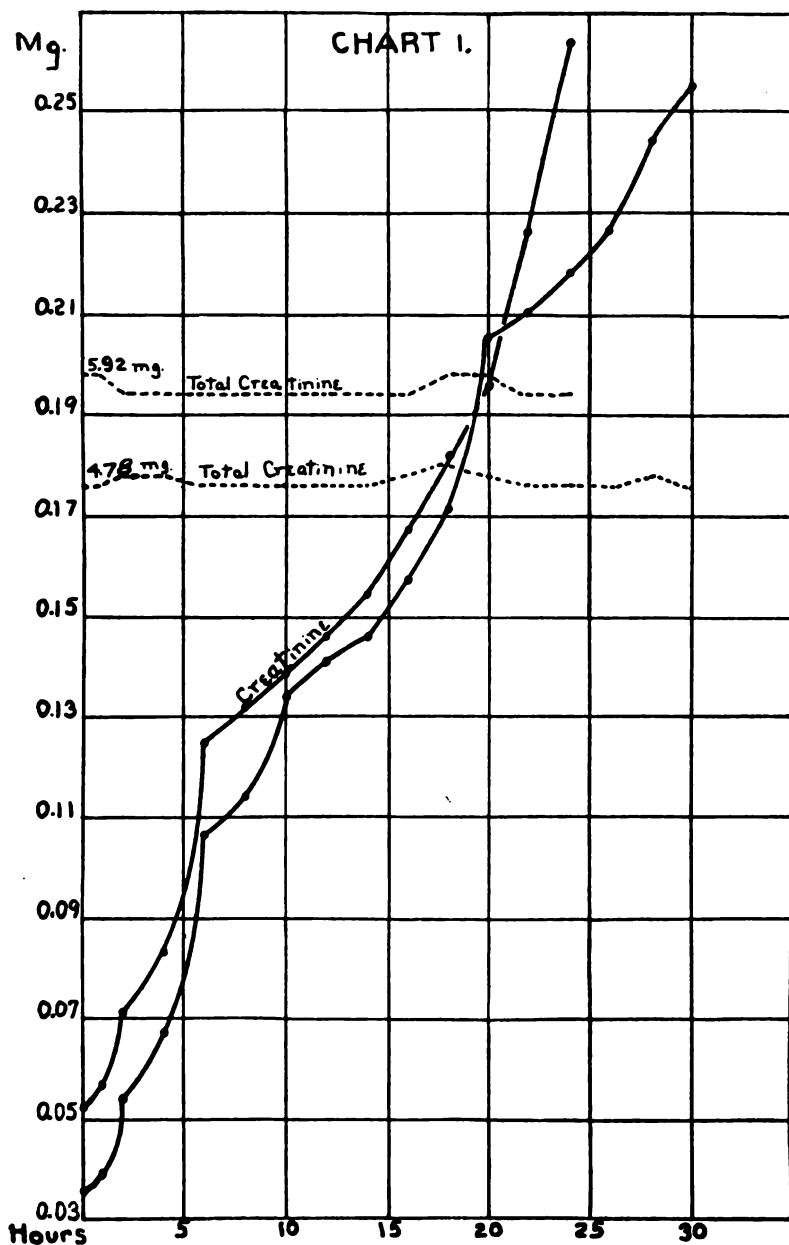
CHART 1. Showing periodicity in rate of creatinine formation during incubation under toluene at 38° at 2 hour intervals for 24 and 30 hours. Changes in total creatinine are also given.

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in gelatin solutions.   They are significant of fundamental phenomena of colloid-containing solutions.

This observation raised the suspicion that the changes in rate of creatinine formation might be due to the changes in the colloid state of the extract.   A study of the amount of material precipitated by uniform centrifugation at 2 hour intervals during incu-

TABLE III.

Showing Changes in Degree of Dispersion of the Colloid System in Muscle Extracts during Incubation at 38°C. as Determined by Centrifugation.

Time in hours.	I	II	III	IV	V
	cc.	cc.	cc.	cc.	cc.
Fresh.	0.07	0.03	0.02	0.04	0.02
1	0.08	0.07	0.08	0.04	0.02
2	0.21	0.16	0.19	0.07	0.07
4	0.40	0.45	0.30	0.35	0.30
6	0.40	0.40	0.30		0.31
8	0.37	0.42	0.30	0.35	0.40
10	0.41	0.40		0.34	0.39
12	0.41	0.39	0.30	0.40	0.39
14	0.37	0.49	0.30		0.39
16	0.40	0.50			
18	0.45	0.50	0.30		
20	0.48	0.50	0.33		
22	0.41	0.52	0.37	0.39	
24	0.50	0.52	0.31		0.41
26			0.33		
28			0.31		
30		0.48			
48	0.52	0.50	0.33		0.49
72	0.68	0.47			0.49
96	0.68	0.53	0.33		
120	0.71		0.38		
168	0.70	0.63			

bation was made, using 5 cc. lots of extract.   The results are given in Table III.   They show that changes take place in the amount of precipitable matter of muscle extracts during incubation.   It is evident that in the first 24 hour period the number and extent of these changes are greater than in later periods.   The equilibrium which are established after each change tends to be fairly stable for increasing periods.   These facts can be correlated

with the changes in rate of creatinine formation since the perio-
dicity is only observed during the first 24 hours. Usually by 30
hours the course of the curve becomes regular. Moreover, it
can be seen from Chart 1 that the early changes in rate of creati-
nine formation are in short periods, and that the periods tend to
lengthen with time. A third correlation which is suggestive,
is that in general the points where the changes in amounts of pre-
cipitable material occur are associated with the points where
abrupt changes are taking place in rate of creatinine formation.
Further proof that the hypothesis expressed above is well founded
was obtained when the muscle extracts were deproteinized and
then incubated.

Attempts at deproteinization with animal charcoal were un-
satisfactory because of adsorption of creatinine and creatine.
The writer, therefore, decided to attempt removal of the colloids
from the extract by boiling, because it was hoped that a study
of the creatinine formation in boiled extract would also show
whether the reaction is catalyzed by an enzyme or not.

400 cc. of undiluted extract were prepared and divided into two
lots of 200 cc. each. To one lot there were added 200 cc. of
Tyrode's solution and 16 cc. of the buffer mixture. The second
lot was delivered in a thin stream from a pipette into 150 cc. of
boiling Tyrode's solution, which was maintained at the boiling
point throughout the addition of the extract and boiled for 1
minute after all the extract had been added. The whole was then
rapidly cooled in ice-cold water, transferred to a graduated cyl-
inder, made to 400 cc. with Tyrode's solution and 16 cc. of buffer
mixture were added. The solution was then mixed by shaking
and filtered through glass-wool. When 5 cc. lots of the boiled
and unboiled extract were incubated it was found that the rate
of creatinine formation during 24 hours as determined at 2 hour
intervals, was slightly greater in the former than in the latter.
Moreover, the boiled extract did not show the periodic changes
in rate of creatinine formation shown in the unboiled extract. The
total creatinine content of the two sets of extract was the same.
The creatinine was slightly increased by boiling.

However, it seemed possible that there might be produced
catalyzing agents in the boiled Tyrode's solution from the well
known destructive action of alkalies on glucose. Consequently a
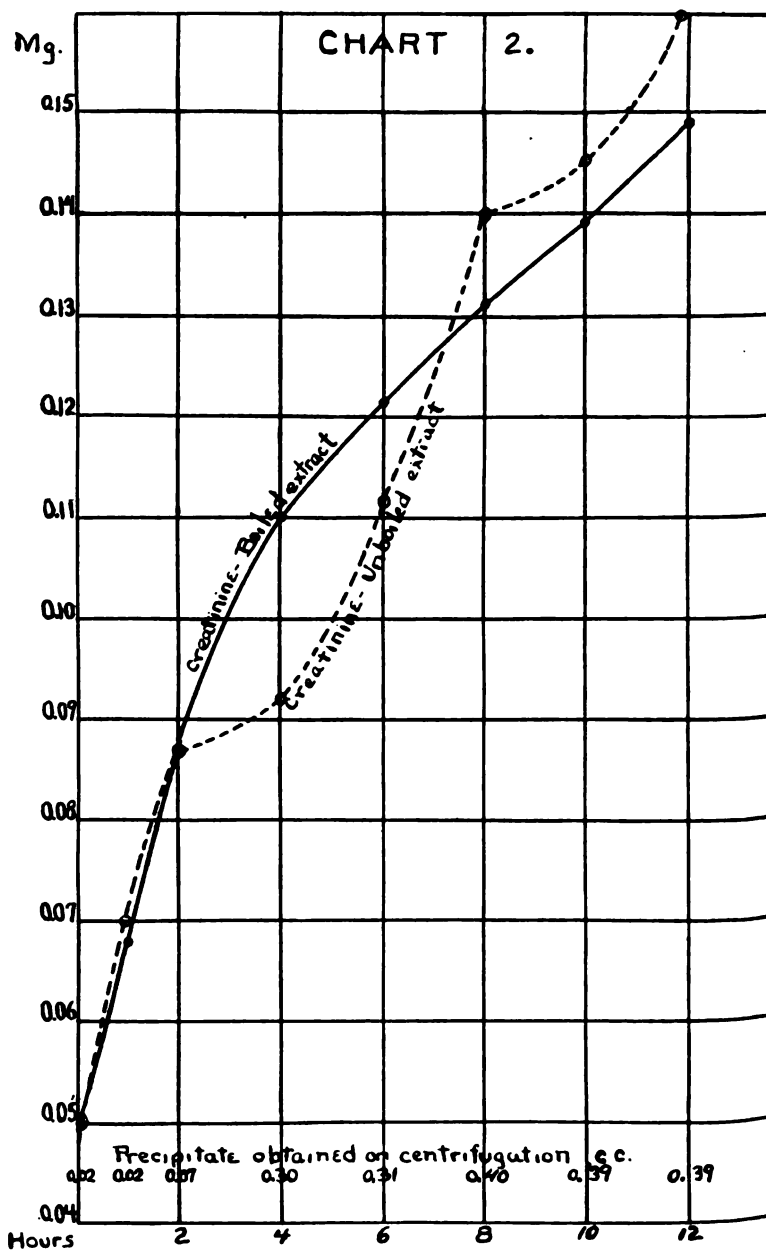
CHART 2. Comparison of the creatinine in boiled and unboiled muscle extracts when incubated under toluene for 12 hours at 38°. Analyses were made every 2 hours. Also record of the precipitate obtained by centrifugation of 5 cc. samples of unboiled extract.

lot of 400 cc. of extract was prepared using, this time, distilled water instead of Tyrode's solution as the diluent for the macerated tissue. The procedure described in the preceding experiment was followed, save that the diluent for the unboiled extract and the extract which was boiled was 0.1 per cent Na_2CO_3. After the boiled sample had been cooled and made to the desired volume, there were added to both the boiled and the unboiled extracts the amounts of salts equivalent to those present in an equivalent volume of Tyrode's solution. After buffering, 5 cc. lots of each

TABLE IV.

Effect of Boiling on the Formation of Creatinine from Creatine in Muscle Extracts Incubated at 38°C.

Hours.	I					II				
	Creatinine.			Total creatinine.		Creatinine.			Total creatinine.	
	Boiled.	Unboiled.	Difference.	Boiled.	Unboiled.	Boiled.	Unboiled.	Difference.	Boiled.	Unboiled.
	mg.	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*
Fresh.	0.042	0.031	35.6	4.39	4.37	0.045	0.034	32.4	4.67	4.63
12	0.164	0.124	32.4	4.39	4.37	0.156	0.135	15.5	4.57	4.63
24	0.226	0.233	− 5.3	4.41	4.37	0.232	0.241	− 6.4	4.65	4.65
48	0.371	0.365	2.6	4.37	4.41					
72	0.473	0.480	− 1.4	4.35	4.41					
96	0.588	0.594	− 0.8	4.35	4.41	0.746*	0.744*	1.1	4.63	4.63
120	0.738	0.728	1.4	4.41	4.41					

* Temperature rose to 39°C.

were incubated as usual. The results of the creatinine determinations are plotted on Chart 2. There were no changes in total creatinine.

It is quite evident that the removal of the colloids has done away with the abrupt changes in the rate of creatinine formation. It is also evident that the rate of creatinine formation in the boiled extract runs along with that of the unboiled extract and at times is slightly greater. The course of the reaction in both extracts is practically the same for days thereafter as shown in Table IV.

This series of experiments gave rise to the idea that possibly the cause of the abrupt changes in rate of creatinine formation in the muscle extract might be an adsorption of the creatinine by the colloids.

Therefore, a large amount of extract was prepared. 15 cc. lots were put into a series of test-tubes and 5 cc. lots into another. After incubation for 4 or 6 hours the 15 cc. lots were centrifuged and creatinine and total creatinine determined in 5 cc. lots of the supernatant liquid, the 5 cc. of precipitated material, and the uncentrifuged extracts. The results are given in Table V.

TABLE V.

Absorption of Creatinine by the Colloids Precipitated on Centrifugation of the Muscle Extracts.

Series.	Fresh extract.		Centrifugation after 4 hours incubation at 38°C.							
			Creatinine.					Total creatinine.		
	Creatinine.	Total creatinine.	A In precipitate.	B In supernatant fluid.	Difference between A and B.	C Not centrifuged.	Difference between A and C.	In precipitate.	In supernatant fluid.	Not centrifuged
	mg.		mg.	mg.	per cent	mg.	per cent	mg.	mg.	mg.
I	0.036		0.087	0.081	7.4					
II	0.C38	4.23	0.093	0.080	16.2	0.081	14.8	4.25	4.27	4.30
III	0.039	4.04	0.105	0.085	23.5	0.083	26.5	4.00	4.02	4.02
	Centrifugation after 6 hours incubation at 38°C.									
IV	0.038	4.23	0.122	0.105	16.1	0.105	16.1	4.28	4.30	4.27
V	0.039	4.04	0.156	0.131	19.1	0.125	24.8	4.04	4.05	4.0

It is seen that there is definite adsorption of creatinine but not of creatine. The absolute amount adsorbed is too small to be detected in the values for total creatinine since the delicacy of this method is not of the same order as is that for creatinine.

The results of the foregoing experiments demonstrate that the periodicity in rate of creatinine formation during the first 2 hours of incubation is due to changes in the state of the colloids in the muscle extracts and that adsorption of creatinine may play a significant rôle. They are suggestive in their general physiological relationships. For not only is it true that changes in chemical composition of a colloid-containing solution influence

the state of the colloids therein, but it is also here demonstrated that changes in the state of colloids influence the rate of chemical reaction.

The creatinine formation during a period of 264 hours autolysis was studied. The contents of the tubes were kept constant at 5 cc. by the addition of a few drops of distilled water when necessary. Daily additions of 0.5 cc. of toluene were made. No changes in total creatinine occurred. Thus there is no evidence of there being present in muscle extract any enzyme which de-

TABLE VI.

Velocity Constants $(k = \frac{1}{t} \log e \frac{a}{a-x})$ *of the Formation of Creatinine from Creatine in Muscle Extracts during Incubation under Toluene at 38°C.*

	A			B	
Creatine, *mg*....	$a = 5.48$			$a = 6.78$	
Time (*t*).	Creatinine (*x*).	Constant (*k*).	Time (*t*).	Creatinine (*x*).	Constant (*k*).
hrs.	*mg.*		*hrs.*	*mg.*	
24	0.218	0.00163	24	0.264	0.00163
48	0.331	0.00129	48	0.418	0.00137
72	0.485	0.00138	75	0.550	0.00119
100	0.649	0.00137	96	0.643	0.00110
120	0.743	0.00133	120	0.746	0.00106
144	0.808	0.00123	144	0.831	0.00099
168	0.920	0.00122	168	0.944	0.00098
192	1.000	0.00119	192	1.020	0.00094
216	1.090	0.00116	216	1.110	0.00093
240	1.180	0.00115	243	1.230	0.00093
264	1.300	0.00117	264	1.338	0.00095

stroys or forms creatine or destroys creatinine. The velocity constants of the reaction, creatine-creatinine, are given in Table VI, together with the data from which they were calculated by the formula for monomolecular reactions. While k is originally high it decreases to an approximately uniform value. It is plain that the reaction, in its later stages at least, is monomolecular. It is my belief that the changes in the value of k are due to the disturbing influence of the colloids of the extract.

The writer, therefore, concludes that *there are no enzymes present in or produced by muscle tissue as such, which form or destroy*

creatine or creatinine or affect the transformation of creatine to creatinine.

The formation of creatinine from creatine is, however, a cata- lyzed reaction. It is a type of catalysis that I will call "bio- catalysis." This differentiation is justified by the fact that it is neither enzymatic catalysis, nor catalysis as usually understood, in that the active agent is not an added foreign substance but is the milieu of the living tissue.

Biocatalysis is the non-enzymatic catalysis of a reaction by the milieu afforded by living tissue, in which the soluble organic and inorganic constituents and the state of the colloid material play the significant rôle. It is possible that some of what has been hitherto designated as enzyme activity may be found on closer analysis to be biocatalysis.

With regard to the origin of the urinary creatinine it is quite clear that although no enzyme activity is concerned, muscle tissue may afford a milieu in which the transformation of creatine to creatinine occurs by virtue of the biocatalytic properties of its constituents. Hence the probability that this change occurs i n the organism and gives rise to the urinary creatinine is further supported.

CONCLUSIONS.

1. Creatinine and creatine are easily dialyzable substances.

2. The transformation of creatine to creatinine in muscle extracts is a reaction of the first order which is masked in its early stages by changes taking place in the state of the colloids of the extracts.

3. From the fact that the amount of creatinine formed in dialyzed extracts is but slightly greater than that formed in the dialysates of such extracts, and from the fact that the increased creatinine formation in non-centrifuged extracts as compared with centrifuged extracts on incubation is also small, and from the fact that the rate of creatinine formation in boiled extracts is no less than that of unboiled extracts it is concluded that no enzyme participates in the transformation of creatine to creatinine in muscles.

4. As no changes occur in the total creatinine content of muscle extracts in any of the procedures described it is concluded that

there is no enzyme in muscle tissue which destroys or forms creatine or creatinine.

5. From the fact that the rate of transformation of creatine to creatinine in muscle extract is some three or four times as great as that taking place in water solutions of the pure compound, and from the results of the experiments detailed in this paper, it is clear that muscle tissue provides a milieu particularly favorable for this change. It is suggested that such types of acceleration be designated as biocatalytic in distinction from those brought about by enzymes and those induced by the addition of substance foreign to the organism.

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AN ANALYSIS OF CAMEL'S COLOSTRUM.

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Through the courtesy of Doctor W. T. Hornaday of the New York Zoological Society we were able to obtain for analysis a sample of the colostrum of a Bactrian camel, 2 days after parturition. Since reports of analyses of milks, other than those of the cow and goat, are none too plentiful it seems of possible value to have a published record of this analysis.

Colostrum from Bactrian Camel—2nd Day after Parturition.

Volume of sample...165 cc.
Appearance ....Thick and rich, not yellow but creamy white.
Taste ....Bland, less taste than cow's milk. Absolutely no
  unpleasant odor.
Reaction ......Slightly amphoteric to litmus, acid reaction more
  marked.
Specific gravity...1.038
Fat...7.4 per cent
Sugar...4.2 "   "
Protein...5.4 "   "
  Casein..4.1 "   "
  Albumin.......................................0.5 "   "
  Globulins, etc................................0.8 "   "
Ash...0.893
CaO...0.272
MgO...0.025
P₂O₅..0.318
K₂O...0.164
Na₂O..0.082
Cl..0.128

EFFECTS OF ETHER ANESTHESIA ALONE OR PRECEDED BY MORPHINE UPON THE ALKALI METABOLISM OF THE DOG.*

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The purpose of the experiments to be described was to obtain further light upon the interrelations between the alkali reserve and hydrogen ion concentration of the blood and the sodium and potassium content of the urine during the anesthesia produced by ether alone, as well as by ether and morphine together. Concerning the alkali reserve of the blood during ether anesthesia, in view of data already available, no doubt exists that the bicarbonate becomes lowered in this condition. Furthermore, evidence is accumulating (1) that ether anesthesia increases the hydrogen ion concentration of the blood.

The rate of sodium and potassium excretion by the kidneys in the normal and anesthetized animal is interesting in connection with some of the explanations which have been given to account for the low alkali reserve of anesthesia. If the diminution is due to the neutralization of the bicarbonate by organic acids resulting from incomplete oxidation, some increase in the sodium and potassium content of the urine might be expected. If, on the other hand, the alkali during anesthesia merely leaves the blood for the tissues in a compensatory fashion, to maintain a constant CO_2: $NaHCO_3$ ratio, then one would expect no change in the rate of alkali excretion.

Methods.

Dogs weighing 10 to 15 kilos were employed. In some cases they were maintained upon a uniform diet·consisting of ground

beef heart, crackers, and agar-agar; in other experiments the animals were kept fasting. After two 24 hour control periods blood was drawn and the animal was then anesthetized by placing it in a glass box which could be closed tightly. Air saturated with ether vapor was blown through the box. In this manner the animal succumbed so easily (in 4 to 6 minutes) that there was practically no struggling. As soon as anesthesia was complete the animal was placed upon an operating table, and by intra-pharyngeal administration of ether kept in the first stratum of the third stage of anesthesia. Blood was again drawn immediately and later at the times mentioned. The minimum period of ether administration was 3 hours. The modifications of the procedure in the morphine-ether experiments may readily be understood from the tables.

Blood CO_2 capacity was determined by the method of Van Slyke; hydrogen ion concentration colorimetrically by the method of Dale and Evans (2).

Sodium and potassium in the urine were weighed as the combined sulfates and potassium was then determined in the mixture by the cobaltic nitrite method described by Drushel (3). To obtain the mixed sulfates the urine was ashed by adding it dropwise to boiling sulfuric acid in a Pyrex Kjeldahl flask. The sulfuric acid was then transferred to a 6 inch evaporating dish, and after evaporating the water on a water bath the excess sulfuric acid was driven off with a free flame. The residue was taken up with water and saturated barium hydroxide solution was added until there was no further precipitation. The volume was then made up to 100 cc., centrifuged, and a suitable portion taken for continuing the analysis. To this portion sulfuric acid was added to remove the barium, and after concentrating it on a hot-plate the barium sulfate was filtered off with a Gooch crucible. The filtrate was concentrated, evaporated in platinum, and ignited.

Ether Experiments.

The results of anesthesia with ether alone are presented in Tables I and II (fed dogs) and III and IV (fasted dogs).

H ion Concentration of the Blood.—In Experiments 1, 6, and 8 the hydrogen ion concentration was increased after 2 hours of ether anesthesia; in Experiment 2 no decided increase occurred but there was a tendency in that direction. The changes noted are considerably smaller than those reported by Atkinson and Ets (1). It is worth noting that the induction of the anesthesia itself produced no change in reaction.

TABLE I.

Experiment 1.

Period.	Urine.*				Blood.			
	Condition.	$Na_2SO_4 + K_2SO_4$	Na_2SO_4	K_2SO_4	Jan. 18		pH	CO_2
					a.m.			
1	Normal.	3.65	2.05	1.60	11.45	Blood.	7.50	59
					p.m.			
2	"	3.41	1.85	1.56	12.14	Ether begun.		
3a	Ether.	0.138	0.068	0.07	12.30	Blood.	7.50	45
3a	Calculated to 24 hr. basis.	0.662	0.326	0.336	2.35	"	7.30	41
3b	Part of urine lost.				3.25	"	7.37	
3a + 3b					3.50	" Ether discontinued.		32
4	Normal.	3.24	1.67	1.57				

* Animal catheterized at 11.00 a.m., daily.

TABLE II.

Experiment 2.

Period.	Urine.*				Blood.			
	Condition.	$Na_2SO_4 + K_2SO_4$	Na_2SO_4	K_2SO_4	Jan. 25		pH	CO_2
					a.m.			
1	Normal.	3.12	1.86	1.26	11.35	Blood.	7.38	47
					p.m.			
2	"	2.93	1.45	1.48	12.14	Ether begun.		
3a	Ether.	0.392	0.210	0.182	12.34	Blood.	7.44	42
3a	Calculated to 24 hr. basis.	2.00	1.06	0.920	2.36	"	7.35	
3b	Remainder of day.	3.23	1.66	1.57	2.41	"		32
3a + 3b	Total anesthesia day.	3.62	1.87	1.75	3.35	" Ether discontinued.	7.32	29
4	Normal.	2.93	1.51	1.42				

* Animal catheterized at 11.00 a.m., daily.

TABLE III.

Experiment 6.

Period.	Urine.*				Blood.			
	Condition.	Na_2SO_4 + K_2SO_4	Na_2SO_4	K_2SO_4	Apr. 1		pH	CO_2
					a.m.			
1	Normal.	1.50	0.12	1.38	11.00	Blood.	7.50	47
2	"	0.90	0.07	0.83	11.55	Ether begun.		
					p.m.			
3a	Ether.	0.078	0.041	0.037	12.15	Blood.	7.57	36
3a	Calculated to 24 hr. basis.	0.546	0.283	0.255	2.00	"	7.34	22
3b	Remainder of day.	1.53	0.45	1.08	3.05	" Ether discontinued.	7.28	19
3a + 3b	Total anesthesia day.	1.608	0.491	1.117				
4		0.568	0.00	0.568				

* Animal catheterized at 11.30 a.m., daily.

TABLE IV.

Experiment 8.

Period.	Urine.*				Blood.			
	Condition.	Na_2SO_4 + K_2SO_4	Na_2SO_4	K_2SO_4	May 19		pH	CO_2
					a.m.			
1	Normal.	1.14	0.545	0.595	11.40	Blood.	7.42	
					p.m.			
2	"	1.50	0.646	0.854	12.23	Ether begun.		
3a	Ether.	0.032	0.000	0.032	12.45	Blood.	7.37	30
3a	Calculated to 24 hr. basis.	0.154	0.000	0.154	2.25	"	7.29	24
3b	Remainder of day.	1.90	0.51	1.39	3.30	" Ether discontinued.	7.26	17
3a + 3b	Total anesthesia day.	2.05	0.51	1.54				
4	Normal.	0.598	0.242	0.356				

* Animal catheterized at 11.30 a.m., daily.

Alkali Reserve of the Blood.—This was lowered in all four ether experiments. The decline appears to begin soon after the induction of anesthesia and in advance of the increase in hydrogen ion concentration. The CO_2 decrease occurred earlier than in Carter's (4) experiments.

Sodium and Potassium of the Urine.—During ether anesthesia the rate of sodium and potassium excretion was greatly decreased. The most plausible explanation for this appears to be the anuria which accompanies ether anesthesia (5).

In the postanesthetic period the rate of sodium and potassium excretion was, however, increased, and to such an extent that the total excretion of these alkalies for the experimental day was abnormally high. The latter fact is most apparent in the fasting experiments (Experiments 6 and 8). In these the alkali loss suffered is reflected in the low figures for the 4th day (normal). This may be due to the greater physiological necessity for conserving these two ions than exists in the experiments in which the animals were fed a diet providing an abundance of sodium and potassium. Experiment 6 was performed upon a pregnant animal, near term, and Experiment 8 upon the same animal about 3 weeks after delivery. The tenacity with which sodium is conserved is well shown, in the first instance especially.

A possible explanation for the postanesthetic increase in sodium and potassium excretion is that during the period of anesthesia acid substances are formed and retained in the body as salts until the discontinuance of the ether. The present experiments afford no light on the nature of these acids, or even upon their existence.

Morphine-Ether Experiments.

The results of anesthesia with ether preceded by morphine are presented in Tables V and VI (fed dogs) and VII and VIII (fasted dogs).

Alkali Reserve of the Blood.—Whereas when morphine is administered alone the alkali reserve actually increases (6), this result of our experiments was influenced by the concomitant and opposite action of ether, so that practically no change occurred. Experiment 7 was an exception, a decided diminution in the alkali reserve being noted.

TABLE V.

Experiment 3.

Period.	Condition.	Na$_2$SO$_4$ + K$_2$SO$_4$	Na$_2$SO$_4$	K$_2$SO$_4$	Feb. 2		pH	CO$_2$
					a.m.			
1	Normal.	3.04	1.06	1.98	10.30	Blood.	7.50	59
2	"	2.21	1.36	0.85	11.10	Morphine.		
3a	Ether.	1.80	1.40	0.396	11.55	Blood.	7.40	54
					p.m.			
3a	Calculated to 24 hr. basis.	9.54	7.42	2.10	12.20	Ether begun.		
3b	Remainder of day.	3.07	1.04	2.03	12.35	Blood.	7.40	57
3a + 3b	Total anesthesia day.	4.87	2.44	2.426	2.20	"	7.35	59
					3.30	"	7.45	
					3.42	"　　Ether discontinued.		58

* Animal catheterized at 11.30 a.m., daily.

TABLE VI.

Experiment 4.

Period.	Condition.	Na$_2$SO$_4$ + K$_2$SO$_4$	Na$_2$SO$_4$	K$_2$SO$_4$	Mar.17		pH	CO$_2$
					a.m.			
1	Normal.	3.53	1.90	1.63	10.00	Blood.	7.50	50
2	"	3.62	2.30	1.32	10.55	Morphine.		
					p. m.			
3a	Ether begun.	1.29	0.76	0.53	12.10	Blood.	7.34	53
3a	Calculated to 24 hr. basis.	7.74	4.56	3.18	12.12	Ether begun.		
					12.30	Blood.	7.23	61
					1.50	"	7.33	
					2.30	"	7.20	47
					3.30	"　　Ether discontinued.	7.25	50

* Animal catheterized at 11.30 a. m., daily.

TABLE VII.
Experiment 5.

Period.	Condition.	Na₂SO₄ + K₂SO₄	Na₂SO₄	K₂SO₄	Mar.23	Blood.	pH	CO₂
					a.m.			
1	Normal.	0.83	0.22	0.61	10.00			47
2	"	0.83	0.14	0.69	10.55	Morphine.		
					p.m.			
3a	Ether.	1.10	0.38	0.72	12.15	Blood.		41
3a	Calculated to 24 hr. basis.	6.60	2.28	4.32	12.18	Ether begun.		
3b	Remainder of day.	1.73	0.15	1.58	12.30	Blood.	7.32	40
3a + 3b	Total anesthesia day.	2.83	0.53	2.30	2.30	"	7.32	46
4	Normal.	0.23	0.10	0.13	3.30	" Ether discontinued.	7.34	42

* Animal catheterized at 11.30 a.m., daily.

TABLE VIII.
Experiment 7.

Period.	Condition.	Na₂SO₄ + K₂SO₄	Na₂SO₄	K₂SO₄	Apr. 6	Blood.	pH	CO₂
					a.m.			
1	Normal.	0.523	0.078	0.445	10.00	Blood.	7.39	42
2	"	0.706	0.071	0.635	10.30	Morphine.		
3a	Ether.	1.22	0.398	0.822	11.50		7.37	40
3a	Calculated to 24 hr. basis.	7.32	2.388	4.932	11.53	Ether begun.		
					p.m.			
3b	Remainder of day.	2.10	0.30	1.80	12.10	Blood.	7.40	43
3a + 3b	Total anesthesia day.	3.32	0.698	2.622	2.15	"	7.36	28
					3.20	" Ether discontinued.	7.35	30

* Animal catheterized at 11.30 a.m., daily.

H Ion Concentration of the Blood.—The tendency for the hydrogen ion concentration to increase was less marked than when ether alone was administered; though in Experiment 4, with no diminution of the alkali reserve, there was a lowering of the pH value.

Sodium and Potassium of the Urine.—The increase in the rate of sodium and potassium excretion is striking. The explanation of this reversal of the usual effect of ether is not obvious. Conceivably, the high CO_2 content of the blood, consequent to depressed respiration might result in drawing alkali from the tissues, and if the kidney threshold for alkali was exceeded, sodium and potassium would pass into the urine. The blood alkali is not increased, however, so that this explanation is not a likely one. In this connection the protective effect of alkalies against ether anuria, described by MacNider (5), is of interest, but it is impossible to state at present whether or not the antidiuretic action of ether can be prevented by morphine.

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THE METABOLISM OF SULFUR.

V. CYSTEINE AS AN INTERMEDIARY PRODUCT IN THE METAB-OLISM OF CYSTINE.*

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The ease with which cysteine is oxidized to cystine has resulted in some confusion as to which of these two sulfur-containing amino-acids is present in the protein molecule and is an active constituent of tissues. Cystine is the form which is isolated from proteins on hydrolysis. However, Heffter (1) and later Arnold (2) showed that practically all tissues gave a positive reaction with sodium nitroprusside and ammonia, a test which seemed to be specific for the mercapto group, -SH. Inasmuch as he found this reaction positive with tissue extracts free from protein, Arnold (3) suggested that cysteine was the substance present in all tissues, which was responsible for the nitroprusside reaction.

In consequence of these facts and others, the belief that cysteine was an active constituent of tissues gained credence. Direct proof of its existence in the organism, however, was lacking until Hopkins (4) in 1921 isolated a dipeptide of glutaminic acid and cysteine from tissues, to which he gave the name "glutathione." He considered that this substance was important in tissue oxidation and reduction and that the mechanism of its reaction consisted in a shifting of the equilibrium between cysteine and cystine according to the activities of the cell. He believed it probable that the significance of the occurrence of cysteine as a constituent of a peptide lay in the fact that it was thereby protected from catabolic changes.

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Another line of evidence which indicates that cysteine is an active substance in metabolism is also available. Jaffe (5) and Baumann and Preusse (6) as well as others have shown that when monohalogen derivatives of benzene, notable bromobenzene, are administered to a dog, there is excreted bromophenyl-mercapturic acid, a derivative of cysteine. However, in the absence of protein in the diet, this synthesis does not take place (7). Further work (8) showed that if cystine be injected subcutaneously into dogs on a protein-free diet, administration of bromobenzene gives rise to mercapturic acid. These results were interpreted to mean that in animals whose diets were deficient in protein content, the cystine of endogenous origin does not pass through the same path in catabolism.

It must be borne in mind, however, in considering these results, that the cysteine is produced as the result of a demand for a detoxicating substance and that its formation may be due to a deviation from the normal path of catabolism in response to abnormal conditions; *i.e.*, the introduction of a toxic benzene derivative into the system. A similar question is raised as to the glycuronic acid used for the detoxication of many foreign organic compounds. It is still debatable whether glycuronic acid, if formed from glucose, is a normal decomposition product or a substance originating in a specialized metabolic process for detoxication.

The purpose of the present communication is to report the occurrence of a cysteine derivative in the urine after the administration of a non-toxic derivative of cystine in which complete oxidation of the molecule was prevented by "protecting" the amino group by substitution. The results support the work of other investigators and indicate that cysteine is a normal stage in the catabolism of cystine in the animal body.

In a previous paper (9), it was shown that if the amino group of cystine was protected from deamination by conjugation with phenylisocyanate, the resulting phenyluraminocystine was not oxidized normally and the greater part of the sulfur of the complex was recovered in the urine as unoxidized sulfur. Cystine under similar conditions was completely oxidized and the sulfur appeared in the urine as sulfates.

During the analyses of the urines for total sulfur, it was observed that, on the days of administration of the phenyluramino-

cystine, the addition of the copper reagent of Benedict to the urine resulted in the formation of a grey-black precipitate. Although the color was slightly different, the substance was considered to be copper sulfide. No such phenomena were observed in normal urines. After the completion of the analyses, we returned to the study of this precipitate and found that after a period of 2 weeks to a month, the precipitate with the copper reagent was obtained in small amounts or was completely lacking. This suggested that we might be concerned with a derivative of cysteine which had been oxidized to cystine on standing. A systematic study of the substance was then made.

The phenyluraminocystine, usually in amounts of 1.0 gm., was administered to rabbits either *per os* or subcutaneously in water suspension or as the sodium salt. The urine was collected for 24 hours after the administration of the phenyluraminocystine.

It was observed that the experimental urines gave an intense purple color with sodium nitroprusside and ammonia (1, 2), a reaction not shown by the normal urines, and that it was possible to remove all the material which gave this reaction by extraction with ether.[1] The urine was acidified and filtered or centrifuged to remove any precipitate (of unchanged phenyluraminocystine?) which might be formed. This precipitate did not react with nitroprusside and ammonia. The liquid was then extracted three times with ether (2 volumes of ether to 1 volume of urine). Occasionally some difficulty was experienced because of emulsions, but when these occurred, they were readily broken by centrifugation. The extracted urine gave no reaction with nitroprusside and ammonia although the test for creatinine in which sodium

[1] Quantitative evidence as to the removal of the organic sulfur compound by ether was obtained by analysis of the urine of a rabbit following administration of phenyluraminocystine. Before extraction with ether, the urine contained 0.146 gm. of total sulfur, and 0.088 gm. of unoxidized sulfur (by difference); after extraction, the total sulfur was reduced to 0.085 gm. and the unoxidized sulfur to 0.027 gm. Before extraction the unoxidized sulfur comprised slightly over 60 per cent of the total sulfur, while after treatment with ether only 31 per cent of the total sulfur was present as unoxidized sulfur, a figure which is only slightly higher than the normal for a rabbit on the type of diet fed. This demonstrates that by far the greater part of the "extra" unoxidized sulfur present was in the form of an ether-soluble compound.

hydroxide is substituted for ammonia was still positive. Creatinine does not react with sodium nitroprusside and ammonia.

After the evaporation of the combined ether extracts at low heat, an oily residue remained which did not crystallize readily. This oily material gave a strong nitroprusside test, a fleeting blue color with dilute ferric chloride, and a grey-black precipitate with copper sulfate. The oil dissolved readily in hot water, and on cooling formed an emulsion from which a gummy light brown mass gradually separated on standing in the ice box. When this solution was allowed to evaporate spontaneously in air, after some time, white crystals separated, and neither crystals nor solution gave a positive nitroprusside reaction. These crystals, although obviously impure, were probably phenyluraminocystine

TABLE I.

Experiment No.	Melting point.	Sulfur.
	°C.	per cent
6	162	12.8
63	159	13.9
64	159	12.8
7	159	10.4
72	158	12.7
62	163	
5	155	
Phenyluraminocystine.	159	13.38

since the melting points were similar. Melting points and analyses for sulfur of several of these impure preparations are given in Table I. It seemed probable that we were dealing with phenyluraminocysteine which had slowly been converted into phenyluraminocystine on standing in air.

Since direct isolation and purification of the unknown substance were unsuccessful, a study was made of the insoluble copper salt. This could be obtained as a grey-black precipitate from the neutral or faintly alkaline urine by cautious addition of a 3 per cent solution of copper sulfate. However, an account of the probability of inclusion of other substances in the precipitate, direct precipitation of the copper salt from the urine was considered inadvisable. Accordingly, the urine was extracted with ether as previously described and the ether extract was shaken

with a dilute (1 per cent) solution of copper sulfate.[2]   The grey-black precipitate of the copper salt which separated in the aqueous layer was drawn off from the ether in a separatory funnel, filtered, carefully washed with cold water, alcohol, and ether, and dried at 80°. Some difficulty was experienced in removing the last traces of copper salts from the precipitate. The light grey powder obtained decomposed on heating at 170–171° (uncorrected) and left a small amount of a black residue in the melting point tube.

For the analysis of the copper salt the material was carefully ashed in a platinum crucible, the residue dissolved in nitric acid, and the copper determined iodometrically in the usual manner. Sulfur was determined either by oxidation with Benedict's reagent, as used in urine analysis or by oxidation in a Parr bomb. The results of analysis are given in Table II.

Four copper salts of phenyluraminocysteine are possible.

Of these Formulas I and III and Formulas II and IV have the same percentage composition. Formula III is considered improbable on account of the size of the ring which would be formed. As will be seen from the table it should be easy to distinguish between the two types of salts by the differences in the percentage of copper and in the ratio of copper to sulfur. The difference in the percentage of sulfur is not great. The figures obtained on analysis indicate that the copper salt probably has the structure assigned by Formula I. While the analytical figures are not theoretical, they may be considered satisfactory in view of the difficulty in washing the bulky precipitate.

TABLE II.

Analysis of the Copper Salt of Phenyluraminocysteine.

	Found.		Theory for	
			$C_{10}H_{16}O_3N_2SCu$	$C_{20}H_{22}O_6N_4S_2Cu$
	per cent		*per cent*	*per cent*
Cu	20.49 20.40 } 20.45		21.09	11.64
S	9.74 9.66 } 9.70		10.61	11.82
Cu: S	2.108		1.988	1.015

Evidence as to the nature of the derivative present in the urine after the administration of phenyluraminocystine was obtained in another way. The urine was treated with dilute copper sulfate directly and the precipitated copper salt filtered off and washed. The salt was suspended in water, the copper removed by hydrogen sulfide, and the copper-free filtrate concentrated *in vacuo*. The liquid remaining in the flask was extracted with ether and the ether evaporated at low temperature. An oily liquid which gave a strong nitroprusside reaction remained. This was dissolved in water, allowed to stand at room temperature for 24 hours, hydrochloric acid added to give a concentration of 10 per cent, and the solution boiled vigorously for 1 hour. If the oily residue contained phenyluraminocysteine, this treatment should have converted it to the hydantoin of phenyluraminocystine. The solution was cooled and allowed to stand in the air. Beautiful white needle crystals, resembling those of tyrosine although somewhat larger, separated. Neither crystals nor mother liquor

gave a positive nitroprusside test. The product was twice recrystallized from 50 per cent alcohol. Positive tests for nitrogen and sulfur were obtained with the crystals. The crystals melted at 117° (uncorrected), and when mixed with a pure sample of the hydantoin of phenyluraminocystine which melted at 116.5–117°, gave a mixed melting point of 115.5–116°. Inasmuch as phenyluraminocystine itself is not precipitated by copper.sulfate, this experiment, which has been repeated with results similar to those reported above, furnishes additional evidence that phenyluraminocystine is first broken down to phenyluraminocysteine in the organism of the rabbit and that this latter substance is then excreted in the urine, since further oxidation is prevented by conjugation of the amino group.

4 gm. of phenyluraminocystine were fed to a dog of 12 kilos weight. No increase in the total or unoxidized sulfur of the urine was observed either on the experimental or succeeding days, nor was there present in the urine any substance which reacted with copper sulfate to give a black precipitate. 0.5 gm. of phenyluraminocystine was fed to a man and the urine secreted in the next 12 hours examined. After extraction with ether as already described, and evaporation of the ether extract, the residual oily material gave a positive nitroprusside test, a black precipitate with copper sulfate, and a blue color with dilute ferric chloride solution. We have never failed to obtain evidence of the presence of phenyluraminocysteine in the urine of the rabbit after administration of phenyluraminocystine, regardless of the manner of administration.

Attempts have been made to prepare phenyluraminocysteine by the reduction of phenyluraminocystine with tin and hydrochloric acid. The insolubility of the latter and the ease with which it is converted to the hydantoin in the presence of acid are factors which interfere with the reduction. As yet we have not succeeded in carrying out the reduction satisfactorily. The work is being continued along these and related lines.

SUMMARY.

In the urine of rabbits to which phenyluraminocystine, a nontoxic substance, has been fed, there is present a substance which has been identified as phenyluraminocysteine. This furnishes

evidence that the first stage in the catabolism of cystine is conversion to cysteine, with subsequent deamination and oxidation of the latter. In the case of the phenyluraminocysteine, deamination has been prevented by conjugation and the cysteine derivative is probably excreted as such in the urine.

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THE ADAPTATION OF THE PENTABROMOACETONE METHOD TO THE QUANTITATIVE DETERMINATION OF CITRIC ACID IN THE URINE.[*]

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The determination of citric acid by the pentabromoacetone method[1] depends upon the formation of water-insoluble pentabromoacetone, when citric acid is oxidized by potassium permanganate in the presence of bromine. The quantitative results would, of course, be vitiated by any impurity of the pentabromoacetone.

When McClure and Sauer[2] employed in urine Kunz's[3] modification of the pentabromoacetone method for citric acid determination, they, at times, found a considerable residue upon treating the pentabromoacetone precipitate with acetone, whereas pure pentabromoacetone is readily dissolved in that solvent. They also found a considerable difference, in many cases, between results obtained by the pentabromoacetone method and those obtained by the Salant and Wise[4] method. These findings pointed out the desirability of further study of the pentabromoacetone method as applied to urine, to ascertain factors interfering with its accuracy.

[*] The early part of the work was done in conjunction with Dr. Hugh Macdonald.

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[2] McClure, W. B., and Sauer, L. W., Comparison of pentabromoacetone method and Salant and Wise's method for citric acid determination in urine, *Am. J. Physiol.*, 1922, in press.

[3] Kunz, R., Ueber das Vorkommen und die Bestimmung von Zitronensäure im Weine und den Nachweis der Zitronensäure in Milch, Marmeladen und Frucht-sirupen, *Arch. Chem. u. Mikrosk.*, 1914, vii, 285.

[4] Salant, W., and Wise, L. E., The action of sodium citrate and its decomposition in the body, *J. Biol. Chem.*, 1916–17, xxviii, 27.

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The following experiment suggested a possible source of erro—
Urine was treated with sulfuric acid and an excess of bromin—
shaken, and allowed to stand. This produced a precipitat—
containing bromine, which was only partially soluble in alcoh—
ether, or acetone. The alcohol, ether, and acetone solutions
this bromine precipitate were red-brown in color and becam—
turbid upon addition of water. ·

Since, during the test for citric acid in the urine by the pent—a
bromoacetone method, bromine is liberated and sulfuric ac—id
has been added previously, the conditions are present for t.he
formation of the bromine precipitate which was described in t.he
preceding paragraph. Should this precipitate be produced, at
least a part of it would be present as an impurity in the penta-
bromoacetone precipitate and would to that extent vitiate the
results of the citric acid determination.

In order to free the pentabromoacetone method, as applied to
urine, from an error which might be due to the appearance of this
bromine precipitate, the following procedures were tried: (1)
Separation of the pentabromoacetone from the bromine precipi-
tate; (2) reduction of the amount of bromine precipitate formed;
and (3) a combination of these two procedures.

Separation of the Pentabromoacetone from the Bromine Precipitate.

(*a*) Numerous trials failed to disclose a *solvent* which would
serve to separate the bromine precipitate in its entirety from the
pentabromoacetone precipitate. (*b*) Pentabromoacetone is readily
volatilized by heat. The effect on bromine precipitate of a degree
of heat sufficient to volatilize actively pentabromoacetone was
tested out. To accomplish this, known quantities of bromine
precipitate and pentabromoacetone, each in a porcelain crucible,
were heated simultaneously in the electric oven at temperatures
approximately 100–105°C. and the loss was determined in each
case. Table I shows the results of four such experiments, each
substance was charted separately. The heating was prolonged in
Experiment 4 for the purpose of more rigorously testing the resist-
ance of the bromine precipitate to this degree of heat.

It is seen from Table I that less than 18 per cent of the bromine
precipitate was lost when the temperature was maintained at
approximately 100–105°C., while the pentabromoacetone was

practically all driven off under similar conditions. However, in one experiment, which is not included in Table I, 10.1 mg. of bromine precipitate, without a pentabromoacetone control, were heated at approximately 100°C. for 24 hours with a loss of 2.5 mg.

TABLE I.

Results of Heating Simultaneously Bromine Precipitate and Pentabromoacetone.

Experiment No.	Bromine precipitate used.	Heated.	Temperature (approximately).	Loss.	Percentage lost.
			Results of heating bromine precipitate.		
	mg.	*hrs.*	*°C.*	*mg.*	*per cent*
1	90.5	4½	100	9.5	10.5
2	73.8	5½	105	9.1	12.3
3	86.5	8½	100	10.9	12.6
4	16.8	39½	105	2.9	17.3
			Results of heating pentabromoacetone.		
1	88.6	4½	100	88.2	99.5
2	69.0	5½	105	68.9	99.9
3	70.6	8½	100	70.6	100.0
4	163.2	39½	105	162.9	99.8

TABLE II.

Results of Heating Mixtures of Bromine Precipitate and Pentabromoacetone.

Mixtures.	Used.	Heated.	Temperature (approximately).	Loss.	Remaining.
	mg.	*hrs.*	*°C.*	*mg.*	*mg.*
Pentabromoacetone..................	52.0	9½	100	51.9	1.2
Bromine precipitate.	1.1				
Pentabromoacetone..................	50.3	24	100	51.8	8.7
Bromine precipitate..................	10.2				

or 24.8 per cent. This percentage of loss was unusually high and was encountered only in this one experiment.

Next, mixtures of small quantities of bromine precipitate and relatively large quantities of pentabromoacetone were heated. Table II shows the results of two such experiments.

These results are such as would be expected after consideration of the findings presented in Table I. In the first experiment the loss upon heating the mixtures was about equal to the quantity of pentabromoacetone employed. In the second experiment, where a larger quantity of bromine precipitate was used and longer heating was carried out, the loss was somewhat greater than the amount of pentabromoacetone. This latter finding is readily explainable on the basis of a partial volatilization of the bromine precipitate.

Also determinations of pentabromoacetone were made in duplicate, in two specimens of native urine with heating used as an additional step to the pentabromoacetone method. Table III gives the results of these determinations.

TABLE III.

Pentabromoacetone Obtained from Urine, Using Heat as an Additional Step in the Pentabromoacetone Method.

Normal urine, 50 cc.	Penta-bromoace-tone found.	Residue after heating.	Heated.	Tempera-ture.
	mg.	*mg.*	*hrs.*	*°C.*
Mixed specimen from adults.	12.3	2.9		
	12.6	2.9	22½	100–105
Mixed specimen from children.	30.5	6.4		
	29.7	7.8	18½	100–106

Reduction of the Amount of Bromine Precipitate.

It was found that a preliminary treatment of the urine with charcoal[5] causes a marked diminution of the quantity of the bromine precipitate which is produced when urine is treated with sulfuric acid and an excess of bromine. This was true, whether the urine was boiled with sulfuric acid and charcoal, or whether the unheated urine was rendered alkaline by sodium hydroxide and only shaken with charcoal. This was demonstrated in the following way: From 150 cc. quantities of a specimen of urine, 45 mg. of bromine precipitate were obtained when charcoal was not used, and 3.9 mg. of bromine precipitate when the urine had been previously treated by boiling with sulfuric acid and charcoal

⁵ Blutkohle, Kahlbaum, was used in all the experiments.

and filtered. A different specimen of urine yielded 31.3 mg. of bromine precipitate per 150 cc. without charcoal treatment, and only 0.1 mg. of bromine precipitate after being rendered alkaline with sodium hydroxide and shaken, unheated with charcoal.

Amberg and McClure[6] had found that to boil urine with sulfuric acid and charcoal may cause a considerable loss of citric acid—as much as 27 per cent in a solution containing the equivalent of 30 mg. of citric acid, in the form of its sodium salt, in 50 cc. of water. It was therefore evident that this method of using charcoal was unavailable for our purpose.

However, it was found in this study that the use of charcoal in an unheated alkaline solution caused little or no loss of citric acid. For example, by using 50 cc. quantities in duplicate, from an aqueous solution of sodium citrate containing the equivalent of 50 mg. of citric acid in each 50 cc., 51.3 and 51.0 mg. of citric acid,[7] respectively, were determined. This method of employing charcoal, therefore, serves the purpose quite satisfactorily.

Combination of Reduction in the Formation of Bromine Precipitate and Separation of Pentabromoacetone from the Bromine Precipitate.

In view of the results reported above, it seemed that a combination of a preliminary treatment of the urine by rendering it alkaline, and shaking with charcoal without heating, and of separation of the pentabromoacetone from impurities by volatilization might be advantageous. To test this out, the pentabromoacetone method for citric acid determination with these two steps added[8]

[6] Amberg, S., and McClure, W. B., The occurrence of citric acid in urine, *Am. J. Physiol.*, 1917, xliv, 453.

[7] Throughout this work in computing the amount of citric acid, 5 mg. were added for each 50 cc. of solution tested, because of an undetermined rest which Amberg and McClure[6] had found to exist.

[8] The pentabromoacetone method for citric acid in urine, as modified, is: Approximately 200 cc. of urine rendered alkaline to litmus with 5 per cent NaOH are filtered. To 150 cc. of filtrate 3 gm. of charcoal are added and the mixture is shaken vigorously unheated for 1 minute and filtered. Duplicate specimens of 50 cc. of the filtrate are taken and to each, 1 cc. of dilute H_2SO_4 is added. Bromine vapor is poured in. If the solution remains clear, 10 cc. of 1:1 H_2SO_4 and 3 cc. of 37.5 per cent KBr are added and the solution is heated in a water bath at 50–55°C. for 5 minutes. 20 cc. of 5

was applied to specimens of urine to which known quantities of sodium citrate had been added. Table IV gives the results of these experiments.

TABLE IV.

Results of Duplicate Determinations of Citric Acid, in Urine by the Penta-
bromoacetone Method, Using Preliminary Charcoal Treatment and Separa-
tion of the Pentabromoacetone from Impurities in the Final Precipitate
by Heat.*

Experiment No.	Citric acid.				
	Added.	Recovered.	In native urine (same method).	Net recovered.	Recovered.
	mg.	*mg.*	*mg.*	*mg.*	*per cent*
1	10.0	21.6	10.3	Average 10.7	107.0
	10.0	21.4	11.2		
2	20.0	30.1	10.4	Average 19.6	98.0
	20.0	30.6	11.2		
3	30.0	43.3	13.8	Average 29.9	99.7
	30.0	44.3	14.0		
4	40.0	50.0	12.6	37.4	93.5
	40.0	50.7	10.7	40.0	100.0
5	40.0	53.3	13.2	Average 38.95	97.0
	40.0	51.1	13.3		
6	40.0	54.9	17.0	37.9	94.8
	40.0	56.3	17.0	39.3	98.0
7	50.0	63.3	13.8	Average 48.9	97.8
	50.0	62.4	14.1		

* Throughout the experiments 50 cc. quantities of the solutions being tested for citric acid, were used for each determination and bromine vapor was added instead of bromine water, following the suggestion of Amberg and McClure.[6]

per cent $KMnO_4$ solution are added drop by drop with vigorous shaking. Concentrated $FeSO_4$, acidified with H_2SO_4, is added, slowly with shaking, in amount sufficient to remove the undissolved $MnO_2 \cdot H_2O$ and excess of bromine. The precipitate is weighed in a Gooch crucible, after drying 24 hours in a desiccator. The crucible and contents are now heated over night in the electric oven at 100–105°C. (A much shorter period of heating is usually sufficient but necessitates reheating and an additional weighing to determine whether all the pentabromoacetone has been volatilized off.) After cooling the crucible is again weighed and the loss of weight due to heating is taken as equivalent to the quantity of pentabromoacetone. From the pentabromoacetone, the quantity of citric acid + $1H_2O$ is calculated by multiplying by 0.464 and adding 5 mg., because of an undeterminable fraction.

CONCLUSIONS.

The pentabromoacetone method may be used for the quantitative determination of citric acid in normal urine with satisfactory results, if the unheated urine has been rendered alkaline, previously, by sodium hydroxide, and shaken with charcoal and filtered; and if the final precipitate is heated as a means of separating pentabromoacetone from impurities.

This work was undertaken at the suggestion of Dr. Samuel Amberg to whom I am indebted for much helpful advice.
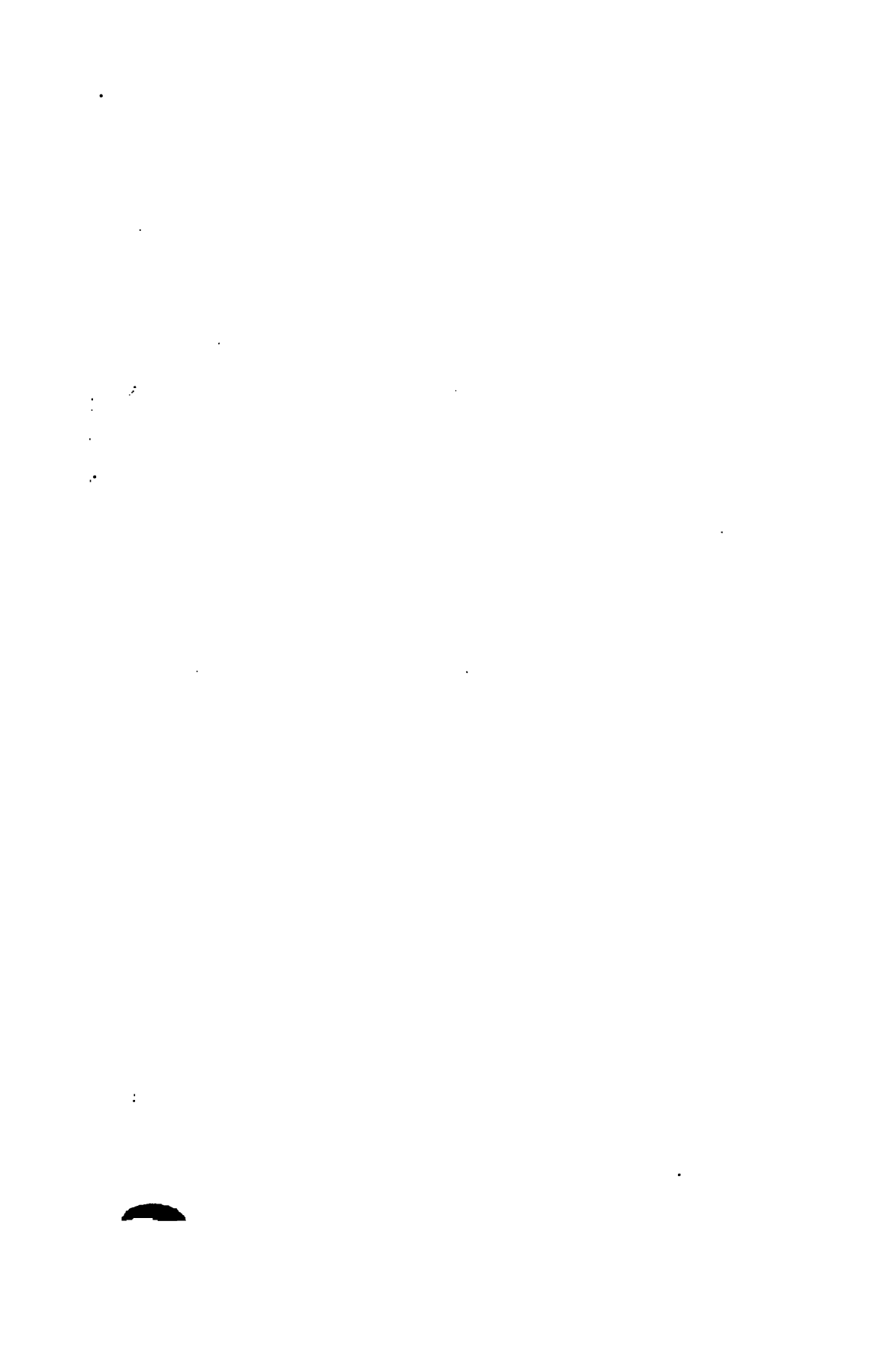
AN ELECTROCHEMICAL STUDY OF THE CONDITION OF SEVERAL ELECTROLYTES IN THE BLOOD.

By B. S. NEUHAUSEN and E. K. MARSHALL, Jr.

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Whether the salts present in the blood have the same degree of ionization as in aqueous solution and whether the various ions are free or bound by the proteins have been two of the questions that have attracted the attention of physiologists and biological chemists. Answers to these questions have been sought principally by two methods—by ultrafiltration of serum with pressure or by compensatory dialysis.

Much of the early work, in which the filtration was performed under a pressure of a number of atmospheres, is open to the criticism voiced by Burian (1) that any labile compounds between the proteins and inorganic ions may have been decomposed by the excessive pressures.[1] Only recently has this difficulty been obviated by Cushny (2) and Richter-Quittner (3) who filtered serum free from the protein at pressures of 150 mm. and lower. Compensatory dialysis while it has a great many advantages is still open to the objection that it is really impossible to include all the other constituents but the one under consideration in exactly the same concentrations inside and outside of the dialyzing sac, which fact might well cause a disturbance of equilibria of the system. It seemed advisable, therefore, to attempt to answer the aforementioned question by a totally different method—the electrometric determination of the concentration of the ions. In this in-

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[1] Bridgman (Bridgman, P. W., *J. Biol. Chem.*, 1914, xix, 511) obtained only a stiffening of egg white with a pressure of 5,000 atmospheres and complete coagulation at 7,000 atmospheres. Lower pressures for extended periods of time would also be effective, particularly if the disruption of these labile compounds is attended with an appreciable decrease in volume.

vestigation, determinations were made of the concentrations of sodium, chlorine, and calcium ions.

Apparatus and Methods.—The sodium ion concentrations were determined by means of a sodium amalgam electrode which is a reversible electrode with respect to sodium ions. In a detailed study of the reliability of the sodium amalgam electrode for determining sodium ions both when sodium salts are present alone and when admixed with salts of other cations, it was shown by Neuhausen (4) that in the range of concentrations of sodium present in the blood, the sodium amalgam electrode is reliable and that, furthermore, potassium and calcium ions in concentrations such as are present in the blood do not interfere. The sodium amalgam was prepared by the electrolysis of a sodium chloride solution. It contained about 0.2 per cent sodium by weight, and was contained in a dropping electrode modified somewhat from the design of Lewis and Kraus (5). This is represented in Fig. 1. The sodium amalgam electrode was connected to a generator of hydrogen and thus there was always an atmosphere of dry hydrogen at a pressure somewhat greater than atmospheric. The amalgam came in contact with the solution at the tip F. By opening the stop-cock C amalgam could be run out and the surface renewed at will. A platinum wire sealed in the glass at P, served as electrical contact between the amalgam contained in the electrode and the mercury in the side arm which was connected to the potentiometer. The rest of the cell arrangement was similar to that of a hydrogen electrode outfit, saturated KCl being used in the bridge and mercury and calomel in saturated potassium chloride being the second half cell. A Leeds and Northrup type K potentiometer and a certified Weston cell were used.

Before making a measurement on blood, the E.M.F. of the Na amalgam electrode against a 0.123 N NaCl solution was determined. From the difference in voltage obtained when the Na amalgam electrode was measured against the NaCl solution and against serum or defibrinated blood, the concentration of the Na ion could be calculated from the formula

$$E = 0.059 \log \frac{0.1026}{c}$$

in which E is the difference in voltages, 0.1026 is the concentration expressed in mols per liter of Na ion in 0.123 N NaCl, and c is the unknown concentration of Na ion in the solution. Since sodium bicarbonate in the concentration present in the blood has
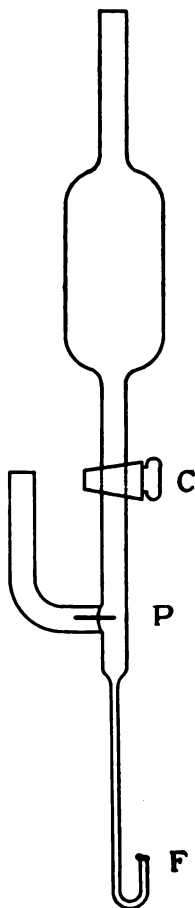
FIG. 1.

a degree of dissociation of about 83 per cent according to Walker and Cormack (6) which is equal to that of 0.12 N sodium chloride, when c is divided by 0.83 the concentration of ionized and union-ized sodium figured as NaCl (and $NaHCO_3$) is obtained.

The chlorine concentration was determined by means of a silver-silver chloride electrode. A silver rod coated with silver chloride is put into the solution in which it is desired to determine the Cl ion concentration. This type of an electrode had been used in the determination of the solubility product of silver chloride and more recently by Brown and Hill (7) in a study of chlorine concentrations in serum. The electrode and solution constitute the positive half cell, while the rest of the cell was made up of a bridge of saturated potassium chloride, and a calomel half cell containing saturated potassium chloride, as in the case of the determination of the concentration of Na ions. The E.M.F. of this electrode in 0.123 N sodium chloride was determined. From the difference in voltage obtained when the electrode was measured against 0.123 N sodium chloride and against serum or defibrinated blood the concentration of the Cl ion can be calculated from the formula

$$E = 0.059 \log \frac{c}{0.1026}$$

in which c signifies the unknown Cl concentration and the other terms have the same significance as in the formula for the computation of the Na ion concentrations. By dividing c by 0.83 the concentration of ionized and unionized chlorine is obtained.

In determining the concentration of the Ca ion a number of difficulties were encountered. Since the calcium amalgam is very reactive, it had to be prepared in a special way as explained by Neuhausen (8) elsewhere. The amalgam of about 0.005 per cent Ca was kept in the electrode under an atmosphere of CO_2, and during the determination of an E.M.F. the amalgam was allowed to flow continuously from the electrode. Otherwise, the arrangement was the same as in the case of the sodium amalgam.

Another difficulty, however, did not prove quite as surmountable as the reactivity of calcium amalgam. When as in the case of a solution such as serum there are present other alkali cations besides the one for which the amalgam is a reversible electrode, there is a tendency for these cations to replace the particular alkali or alkaline earth in the amalgam, so that a mixed electrode is obtained, which is of no use in determining concentrations. The effect is especially marked in the case of serum since the concentration of the sodium is about 100 times as great as that of the calcium.

To obviate this difficulty, solutions were prepared which contained 0.123 N NaCl, 0.03 N NaHCO₃, 0.0025 M CaCl, and 0.0015 M MgCl. Such a solution approximates as far as disturbing effects are concerned the composition of dog's blood, the effect of potassium being included in the sodium. The E.M.F. was determined when the Ca amalgam was dropping into this solution. The E. M. F. was then determined when the amalgam was dropping into serum. From the difference in voltage obtained in the two cases the concentration of the Ca ion can be calculated from the formula

$$E = 0.0295 \log \frac{0.0025}{c}$$

in which c is the unknown concentration of the calcium ion, and the other terms have the same significance as in the formula used above.

It should be noted that the calculation of the calcium concentration is valid only if it is certain that the calcium electrode in the solution approximated in every way the action of the calcium amalgam in serum. This cannot be claimed absolutely, so that an error as large as 25 per cent may be inherent in this method. Other methods are being worked out which may do away with the use of a calcium amalgam electrode for determining the calcium ion.

The sample of blood or serum was obtained from dogs in some cases (6, 7, 8) without the use of anethesia and in others with the aid of paraldehyde or ether. No difference was noticed in the ionic concentration in either case.

Results.—The results obtained in this investigation on serum are given in Table I, under headings which are self-explanatory. The quantitative analysis of the sodium, calcium, and chlorine were made by the Department of Pediatrics of this University through the courtesy of Professor Howland and Doctor Kramer, whose kindness is hereby acknowledged.

On examination of Table I it is apparent that all the sodium and chlorine found in the blood can be accounted for within experimental error on the assumption that the sodium and chlorine in the serum are present as sodium chloride and sodium bicarbonate. This fact is in agreement with the finding of Neuhausen (9) that on the assumption of a similar degree of dissociation for salts in the serum as for aqueous solutions of equal concentration the

observed and calculated lowering in vapor pressure and lowering in freezing point of the serum are in good agreement. These observations in themselves do not rule out the possibility of the presence of Na-protein compounds in the blood, for it could be maintained that the Na-proteinates have the same degree of ionization as the bicarbonate. In fact Rona and György (10) claimed to have confirmed by dialysis Hamburger's (11) contention that about 15 per cent of the total sodium was in an indiffusible form. Against the conclusion of these authors may be cited the results obtained by Cushny who by filtering ox serum free from colloids at a pressure of only 150 mm. of mercury found that all the non-colloid constituents of the serum except Ca and Mg are in simple

TABLE I.

Sample No.	Difference in E.M.F. of Ag-AgCl electrode in sample and 0.123 N NaCl.	Total chlorine normality calculated.	Total Cl normality found by analysis.	Difference in E.M.F. of Na electrode in sample and 0.123 N NaCl.	Total Na normality calculated.	Total Na normality found by analysis.	Difference in E.M.F. of Ca electrode in sample and 0.002 M CaCl₂.	Total Ca molarity calculated.	Total Ca molarity found by analysis.
1	−0.0027	0.1112	0.1117	−0.0066	0.1609	0.1495			
2	−0.0029	0.1103	0.1127	−0.0070	0.1624	0.1586			
3	−0.002	0.1142	0.1140	−0.1169	0.1617	0.1582			
4	−0.0029	0.1103	0.1117	−0.0070	0.1624	0.1586			
5	−0.002	0.1142	0.1145	−0.0065	0.1592	0.1494	+0.020	0.000524	0.0025
6	−0.003	0.1100	0.1071	−0.0060	0.1562	0.1556			
7	−0.0029	0.1103	0.1093	−0.0055	0.1533	0.1491	+0.0193	0.000554	0.00245
8	+0.001*	0.1227	0.1081	−0.0060	0.1562	0.1521	+0.019	0.000567	0.00252

* Some potassium chloride from the bridge flowed over.

solution in the blood. Richter-Quittner (3) found that all the sodium could be filtered from serum at low pressures. These facts indicate that it is unlikely that there is any appreciable quantity of bound sodium in the serum. Evans (12) has shown by a careful calculation that at a pressure of 40 mm. CO_2 there is no margin left from the total cations present to combine with the proteins. In this work it was found that the concentration of sodium ions does not change more than 1 to 2 per cent when the pressure of CO_2 is varied from 0 to 40 mm. The sodium apparently is not bound to any appreciable extent.

Most investigators agree at present that only a small portion, if any, of the chlorine in the serum is in a bound or non-diffusible

form. This binding observed in diffusion is dependent on the pH of the solution and can be accounted for on the basis of a Donnan membrane equilibrium existing on the two sides of the filter. Because of the greater quantity of chlorine found in plasma when the ashing method is used than when the precipitation of the proteins is used, Falta and Richter-Quittner (13) postulated the existence of a compound of fibrinogen and chlorine. No such difference appears in the use of the two methods in the analysis of serum. These observed differences in plasma have been partly explained, on the difference of colloidal condition, so that these authors have recently declared (14) that the existence of the fibrinogen-chloride compound is an open question. Van Creveld (15), however, has proved by analysis of the aqueous humour, which may be considered as a dialysate of blood, that there is actually more total chlorine in the aqueous humour obtained on the first puncture which is practically protein-free than in the liquid of the second puncture which contains 2 to 5 per cent protein. Van Creveld concludes, therefore, that no appreciable quantity of chlorine is bound. Rona (16) by compensatory dialysis and Asher and Rosenfeld (17) by diffusion of serum against water concluded that all of the chlorine is present in a diffusible form. Cushny and Richter-Quittner have both found, moreover, that all of the chlorine can be completely filtered at low pressures. The results in Table I bear out the contention of those who have claimed that no appreciable quantity of the chlorine is bound. It was found, moreover, that the pressure of CO_2 had no influence on the chlorine concentration in serum. In defibrinated blood slight variations were observed with varying CO_2 pressure. As both serum and whole blood can be used with the sodium amalgam and silver chloride electrodes, these methods are feasible for clinical investigations.[2]

From the results in Table I it appears that about 80 ± 5 per cent of the calcium is present in some unionized form. All in-

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[2] When defibrinated blood is used, it should be remembered that the concentration of the sodium ion in the serum is determined. Since there is less sodium in the corpuscles than in plasma, results by quantitative analysis would be lower than those calculated from the electrometric determination. Incidentally a combination of the electrometric determination and quantitative analysis would indicate any abnormal distribution of sodium between corpuscles and serum.

vestigators have agreed that only a portion of the calcium is free. Rona and Takahasi (18) found by dialysis that 75 per cent of the Ca is diffusible. Cushny found that 60 to 70 per cent of the Ca is filterable, while Richter-Quittner (3) who filtered at even lower pressures found only 5 to 7 per cent of the total calcium was filtered. The results in the table are apparently midway between the results of Cushny and Richter-Quittner. From the results of the table it is apparent that there are no grounds for assuming the existence of any ion-protein combination for sodium or chlorine, but that a calcium-protein compound is very probable.

SUMMARY.

The sodium, chloride, and calcium ion concentrations in blood have been determined by suitable electrodes. (A comparison of the ionic concentrations found with the total concentrations as determined by ordinary analytical methods indicate that the sodium and chloride are present as in an aqueous solution of sodium chloride (and sodium bicarbonate) of the same concentration, while only about 10 per cent of the total calcium is present in ionic form.

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A NOTE ON THE DETERMINATION OF URIC ACID.

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In a recent paper[1] we described a reagent which under given conditions would give, with uric acid a far greater color than was developed by the older Folin reagent. The preparation of this reagent was time-consuming and somewhat complicated.

We have found a very simple method by which the same result can be obtained. The "B" salt is prepared as described in the previous paper. This dry solid is dissolved in 95 per cent alcohol (about 250 cc. of alcohol for each 100 gm. of solid). A residue of simple phosphates remains undissolved. The solution is filtered, (more alcohol may be added if the solution does not filter readily), and evaporated to dryness in a water bath, with frequent decolorizations with bromine water. The dry product is dissolved in a little hot water, decolorized again, and evaporated once more to dryness.

A 20 per cent solution is now made of this purified "B" solid—accurate to about 1 per cent. To each 100 cc. of this solution are added 34 cc. of an exactly 2.5 per cent water solution of primary calcium phosphate (Baker analyzed c. p.).

This final solution may now be used in the same manner as the more complicated reagent previously described.

The same turbidity will develop in the final colored solution and will clear up in the course of a few minutes, after which the solution will remain clear for several hours. If the flocculent precipitate does not entirely disappear, a little more 20 per cent phosphotungstate "B," as prepared above, may be added. If the crystalline precipitate should develop—in our experience it has not—more calcium phosphate should be added.

The rest of the procedure is as before described.

[1] Jackson, H., Jr., and Palmer, W. W., *J. Biol. Chem.*, 1922, l, 89.

CALCIUM AND PHOSPHORUS METABOLISM IN CHILDHOOD.*

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Previous publications from this laboratory (1, 2) have reviewed the literature dealing with the calcium requirement of the adult and have offered what appears to be a satisfactory estimate of the average amount of this element required for the maintenance of equilibrium in the normal man or woman; viz., 0.45 gm. per day per 70 kilos of body weight.

The present paper describes experiments designed to determine the rate of storage of calcium in normal children of different ages and the nature and amount of the intake required to support optimum calcium storage in the growing child.

In the interest of brevity we omit a historical review which has been summarized elsewhere (1, 3) and any attempt to extract the data relating to calcium metabolism from the recent and rapidly growing literature of rickets and related diseases of the bones inasmuch as the present article treats only of the normal metabolism. The outstanding importance of calcium in the food requirements of growth has been strikingly demonstrated upon laboratory animals by Osborne and Mendel (4) and by McCollum, Simmonds, and Parsons (5). The results of previous investigations in which calcium storage has been measured in normal children are summarized with references in Table V. See also the

work of Hoffström (6) on the metabolism and storage of calcium in a pregnant woman.

The experiments here described were carried out in four series. The purpose of the first series was to study the relation of calcium retention to age. Twelve children from 3 to 13 years old were studied as to balance of intake and output of calcium and phosphorus during a period of 9 days. All the children were normal and received a normal mixed diet including a fixed allowance of 750 gm. of milk per child per day. This resulted in a nearly uniform calcium intake of about 1 gm. per child per day. The calcium retention varied from 0.15 to 0.62 gm. per day, increasing with the age and size of the child. Calculated to the basis of size the results show fair uniformity and indicate an average daily storage of 0.01 gm. of calcium and 0.008 gm. of phosphorus per kilo of body weight per day in normally growing children of 3 to 13 years of age. In the second series of experiments three of the children who had served as subjects during the first series were kept under continuous control and observation with quantitative determination of intake and output of calcium and phosphorus for 48 days—a series of eight experiments of 6 days each—the calcium intake being varied from period to period by systematic changes in the amount of milk in the diet, in order to determine what daily allowance of milk would induce optimum storage of calcium in the growing organism of the child. The three children studied in this series were 4, 5, and 12 years of age, and in each case it was found that optimum storage of calcium required an allowance of about a quart (750 or 1,000 gm.) of milk per child per day. Combining the data of both series it is found that the average storage of calcium was 70 per cent higher (0.017 gm. as against 0.010 gm. per kilo of body weight per day) when the daily allowance of milk was increased from 750 to 1,000 gm. per child per day.

When the food intake included 1,000 gm. of milk per day, the other foods of the mixed diet being taken *ad libitum*, the daily intake of calcium by these normally growing children of 4 to 12 years averaged 0.053 gm. of calcium per kilo of body weight, and of this intake approximately one-third was retained in the growing body.

The experiments of the third and fourth series were designed to determine whether children utilize the calcium of vegetables as

well as they do that of milk. In Series III the same three children were used as in Series II and calcium and phosphorus balances were determined continuously for 27 days divided into three experiments of 9 days each. During the first and third of these periods each child received 500 gm. of milk per day as the only calcium-rich food of a mixed diet, while during the second period there were added to the diet such amounts of carrots and spinach as would furnish the quantity of calcium which would have been supplied by a second 500 gm. of milk. Had the calcium of the vegetables been utilized as efficiently as that of milk, this would have been equivalent to increasing the daily allowance of milk from 500 to 1,000 gm. and a distinct increase of calcium storage would have resulted as shown by the experiments of Series II. This was found not to be the case. In the final series of experiments three different children were used and the method of comparing the utilization of the calcium of the vegetables with that of milk was modified by using 750 gm. of milk per day in the first and third period while during the second period one-half of this milk was replaced by enough of the vegetables to furnish the same amount of calcium. Here again it was found that the children utilized the calcium of milk to better advantage than they did the calcium of the vegetables. The writers entertain no doubt as to the desirability of a liberal use of vegetables in the feeding of children, but the vegetables should be used in addition to a liberal allowance of milk and should not be allowed to reduce the amount of milk consumed.

EXPERIMENTAL DATA AND DISCUSSION.

First Series.

The purpose of this series of experiments was to obtain data which in conjunction with the data already on record would serve to establish quantitatively the extent to which calcium is stored in the bodies of healthy growing children of different ages under ordinary normal conditions of diet. Twelve healthy, well developed children from 3 to 13 years of age were studied. Inasmuch as the data recorded by previous investigators were chiefly for boys (7, 8), a larger number of girls than of boys were included in the present study. The twelve children were divided into three groups.

Group I consisted of four children 3 to 6 years of age.   Their diet was milk, bread, butter, oatmeal, orange juice, and potato.

Group II comprised four girls 6 to 10 years old, who received milk, bread, butter, potato, oatmeal or macaroni, orange juice, and apple.   Small portions of ice-cream were also given; once during the first, and once during the third period.

Group III consisted of four girls 10 to 13 years old.   The diet for this group was milk, bread, butter, oatmeal, orange juice, apple, potato, and beef.

As the milk was the chief source of calcium in all cases (as in most dietaries), and it was desired to insure a fairly regular intake of calcium at about the level to which the children were accustomed, the daily allowance of milk was fixed at 750 gm. each for ten of the children; 600 and 500 gm., respectively, for the remaining two. The rest of the diet, which consisted of foods of much lower calcium content, was, in general, partaken of according to the taste and appetite of the individual.

Table I shows the average food intake of each child in grams per day of the food as served at the table.   All food was weighed, sampled, and analyzed for calcium and phosphorus.   The energy and protein values of the food were calculated from the values given in Rose's Laboratory Handbook for Dietetics.

The children (in groups of four as indicated above) were housed throughout the experimental periods in rooms devoted exclusively to this purpose where they were under the constant supervision and care of one of the writers (E. H.) for 1 preliminary day and 9 consecutive experimental days.   The 9 experimental days were divided into three periods of 3 days each.   The urine and feces of each child were collected quantitatively for each period (carmine being used to mark the beginning and end of each period in the feces), and were subsequently analyzed for calcium by the McCrudden method and for phosphorus by the gravimetric method of double precipitation first as ammonium phosphomolybdate and subsequently as magnesium ammonium phosphate.

Table II shows in grams per day the average intake, output, and balance of calcium and phosphorus for each child during each 3 day period, and Table III shows the daily intake, output, and balance for each child as averaged for the 9 experimental days treated as one period.   As the balances for the successive periods

show no distinct trend upward or downward, it appears probable that calcium intakes obtaining in these experiments were in general not very different from those to which the children were accustomed; that such fluctuations from period to period as are shown by the data of Table II in the cases of some individual children are most probably due to chance variations of output; and that average balances shown by Table III afford the best available indication of the amounts of calcium and phosphorus ordinarily stored in normally growing children of 3 to 13 years of age.

It will be seen that with calcium intakes of 0.65 to 1.02 gm. per day, the balances ranged from +0.15 to +0.62 gm. per day. The amount stored does not in these cases run parallel with the intake, as between the different children, but shows a closer relationship to the age and size of the child. In the case of phosphorus the balance ranges from +0.09 gm. on an intake of 0.80 gm., to +0.53 gm. on an intake of 1.46 gm., the storage here showing a direct relationship both to the intake and to the body weight.

Table IV shows the average daily storage of calcium and of phosphorus by each child calculated in terms of body weight. It will be seen that under the dietary conditions of these experiments the children of all ages from 3 to 13 years show a fairly constant storage of about 0.01 gm. of calcium per kilo of body weight per day; while the storage of phosphorus averages 0.008 gm. per kilo of body weight, but with much larger variations among the individual children.

In Table V are summarized the data of the present series and of all similar determinations of calcium and phosphorus balances of ormal children which we have found in the literature, the data being calculated to grams of element stored per day per kilo of body weight and averaged in groups according to age. From these data it appears that normal storage per kilo of body weight is highest in the second half of the first year, when it averages nearly 0.04 gm. of calcium and nearly 0.03 gm. of phosphorus; at the age of 3 to 8 years the average rate of storage has become about 0.01 gm. each of calcium and phosphorus per kilo of body weight per day and continues at nearly this rate throughout the following years of rapid growth and development.

TABLE I.

Average Daily Food Intake of Each Child in Grams.

Name.	Age.		Weight.	Milk.	Bread.	Butter.	Orange juice.	Sugar.	Oatmeal.	Potato.	Apple.	Ice-cream.	Calories.	Protein.
	yr.	*mo.*	*kg.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Period I.														
K. C.	3	7	16.2	750	198	16.5	83	8	190	117			1,402	51
C. B.	3	8	15.2	757	129	7.7	55	3	110	100			1,074	42
M. O.	4	7	19.5	736	175	15.3	83	9	167	133			1,319	48
A. B.	6		21.4	750	265	16.7	83	10	300	167			1,678	61
M. P.	6		19.1	602	196	16.7	52	5	18	150	46	26	1,240	42
R. G.	7	4	25.2	755	127	15.6	55	10	100	133	104	22	1,244	43
R. B.	8	9	39.3	557	163	16.7	55	3	50	200	80	28	1,198	40
R. T.	9	6	33.0	702	121	9.7	55	3	50	167	107	30	1,137	40
												Meat.		
L. M.	10	11	35.4	750	413	26.6	82	10	167	273	194	67	2,412	87
A. F.	11	5	34.2	749	247	26.6	82	10	216	256	187	62	1,991	72
E. C.	11	7	30.7	750	302	26.6	82	10	83	256	172	63	2,039	73
L. G.	13	6	54.9	750	510	26.6	79	3	17	273	166	62	2,501	91
Period II.														
K. C.	3	7	16.2	750	150	13.5	85	8	200	128			1,269	47
C. B.	3	8	15.2	749	245	1.0			150	67			1,296	53
M. O.	4	7	19.5	750	245	15.0	57	9	167	83			1,409	54
A. B.	6		21.4	750	264	15.0	85	10	417	150			1,731	64
									Macaroni.					
M. P.	6		19.1	600	216	26.7	78	30	131	200	1		1,681	52
R. G.	7	4	25.2	733	175	18.7	82	30	141	133	126		1,635	53

R. B.	8	9	39.3	467	162	17.7	82	30	140	123	134		1,455	43
R. T.	9	6	33.0	733	195	15.2	82	30	157	199	157		1,784	59
									Oatmeal.			Meat.		
L. M.	10	11	35.4	750	492	28.7	80	35	233	190	136	97	2,658	100
A. F.	11	5	34.2	750	383	30.3	83	35	267	190	176	97	2,439	91
E. C.	11	7	30.7	750	435	28.7	83	35	200	190	130	97	2,486	94
L. G.	13	6	54.9	750	809	28.7	83	35		190	132	97	3,293	123

Period III.

K. C.	3	7	16.2	750	223	15.0	85	8	267	150			1,525	55
C. B.	3	8	15.2	750	234	1.0	24	5	283	117			1,396	56
M. O.	4	7	19.5	750	309	15.0	85	9	283	150			1,761	64
A. B.	6		21.4	750	357	15.0	85	10	383	200			1,976	72
									Macaroni.			Ice-cream.		
M. P.	6	4	19.1	600	182	23.3	76	0	169	89	53	26	1,528	52
R. G.	7	9	25.3	748	196	23.3	80	0	171	150	117	27	1,753	59
R. B.	8	6	39.3	500	279	23.3	80	0	199	196	117	30	1,868	61
R. T.	9		33.0	750	341	25.0	80	0	187	238	88	29	2,197	75
									Oatmeal.			Meat.		
L. M.	10	11	35.4	745	485	31.7	82	10	200	240	152	97	2,672	100
A. F.	11	5	34.2	750	361	33.0	82	10	300	240	175	97	2,447	91
E. C.	11	7	30.7	750	434	31.7	82	10	200	223	156	97	2,542	95
L. G.	13	6	54.9	750	764	33.3	82	0	200	223	141	97	3,236	119

TABLE II.
Calcium and Phosphorus Storage during First Series of Experiments, in Grams per Day

Name.	Sex.	Age.		Weight.	Calcium.				Phosphorus.			
					Intake.	Urine.	Feces.	Balance.	Intake.	Urine.	Feces.	Balance.
		yr.	mo.	kg.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Period I												
K. C.	F.	3	7	16.2	0.883	0.025	0.775	+0.084	1.029	0.531	0.407	+0.091
C. B.	M.	3	8	15.2	0.859	0.015	0.696	+0.148	0.917	0.465	0.326	+0.125
M. O.	F.	4	7	19.5	0.862	0.010	0.598	+0.254	0.990	0.497	0.388	+0.105
A. B.	M.	6		21.4	0.914	0.107	0.628	+0.179	1.168	0.668	0.432	+0.068
M. P.	F.	6		19.1	0.738	0.048	0.413	+0.277	0.827	0.613	0.204	+0.010
R. G.	"	7	4	25.2	0.892	0.051	0.544	+0.297	0.955	0.500	0.343	+0.112
R. B.	"	8	9	39.3	0.693	0.015	0.288	+0.390	0.800	0.532	0.168	+0.100
R. T.	"	9	6	33.0	0.837	0.043	0.518	+0.276	0.889	0.517	0.316	+0.056
L. M.	"	10	11	35.4	0.976	0.072	0.605	+0.299	1.325	0.682	0.407	+0.236
A. F.	"	11	5	34.2	0.940	0.026	0.557	+0.357	1.205	0.653	0.461	+0.091
E. C.	"	11	7	30.7	0.939	0.032	0.459	+0.448	1.173	0.594	0.329	+0.250
L. G.	"	13	6	54.9	0.979	0.033	0.470	+0.476	1.311	0.448	0.355	+0.508
Period II.												
K. C.	F.	3	7	16.2	0.873	0.016	0.733	+0.124	0.999	0.578	0.376	+0.045
C. B.	M.	3	8	15.2	0.872	0.028	0.732	+0.112	1.008	0.535	0.332	+0.141
M. O.	F.	4	7	19.5	0.886	0.011	0.740	+0.135	1.038	0.506	0.419	+0.113
A. B.	M.	6		21.4	0.925	0.108	0.573	+0.244	1.231	0.658	0.456	+0.117
M. P.	F.	6		19.1	0.734	0.024	0.522	+0.188	0.884	0.475	0.222	+0.187
R. G.	"	7	4	25.2	0.871	0.055	0.617	+0.199	0.968	0.572	0.361	+0.035
R. B.	"	8	9	39.3	0.582	0.029	0.370	+0.183	0.708	0.520	0.202	−0.013
R. T.	"	9	6	33.0	0.881	0.039	0.631	+0.211	1.016	0.449	0.408	+0.159
L. M.	"	10	11	35.4	0.991	0.093	0.601	+0.297	1.419	0.526	0.441	+0.452
A. F.	"	11	5	34.2	0.974	0.044	0.696	+0.234	1.353	0.473	0.444	+0.436
E. C.	"	11	7	30.7	0.975	0.051	0.514	+0.410	1.350	0.527	0.353	+0.470
L. G.	"	13	6	54.9	1.039	0.068	0.261	+0.710	1.547	0.602	0.226	+0.719
Period III.												
K. C.	F.	3	7	16.2	0.899	0.013	0.540	+0.346	1.107	0.521	0.319	+0.267
C. B.	M.	3	8	15.2	0.891	0.028	0.683	+0.180	1.100	0.532	0.355	+0.213
M. O.	F.	4	7	19.5	0.921	0.015	0.748	+0.158	1.190	0.418	0.519	+0.253
A. B.	M.	6		21.4	0.946	0.126	0.621	+0.199	1.306	0.707	0.462	+0.137
M. P.	F.	6		19.1	0.751	0.030	0.497	+0.224	0.839	0.537	0.182	+0.120
R. G.	"	7	4	25.2	0.920	0.066	0.483	+0.371	1.017	0.515	0.254	+0.247
R. B.	"	8	9	39.3	0.687	0.018	0.373	+0.296	0.887	0.442	0.251	+0.193
R. T.	"	9	6	33.0	0.965	0.043	0.590	+0.332	1.176	0.393	0.406	+0.377
L. M.	"	10	11	35.4	0.985	0.056	0.618	+0.311	1.408	0.771	0.409	+0.228
A. F.	"	11	5	34.2	0.974	0.028	0.607	+0.339	1.371	0.640	0.398	+0.333
E. C.	"	11	7	30.7	0.979	0.043	0.526	+0.410	1.365	0.711	0.322	+0.332
L. G.	"	13	6	54.9	1.032	0.116	0.237	+0.679	1.522	0.992	0.175	+0.355

TABLE III.

Average Calcium and Phosphorus Storage for the 9 Days of the First Series, in Grams per Day.

Name.	Age.		Weight.	Calcium.			Phosphorus.		
				Intake.	Output.	Balance.	Intake.	Output.	Balance.
	yr.	mo.	kg.	gm.	gm.	gm.	gm.	gm.	gm.
K. C.	3	7	16.2	0.885	0.700	+0.185	1.045	0.910	+0.135
C. B.	3	8	15.2	0.874	0.727	+0.147	1.009	0.848	+0.161
M. O.	4	7	19.5	0.890	0.707	+0.183	1.073	0.916	+0.157
A. B.	6		21.4	0.928	0.721	+0.207	1.235	1.128	+0.107
M. P.	6		19.1	0.741	0.511	+0.230	0.850	0.744	+0.106
R. G.	7	4	25.2	0.894	0.605	+0.289	0.980	0.848	+0.132
R. B.	8	9	39.3	0.654	0.364	+0.290	0.798	0.705	+0.093
R. T.	9	6	33.0	0.894	0.621	+0.273	1.028	0.830	+0.198
L. M.	10	11	35.4	0.984	0.682	+0.302	1.384	1.079	+0.305
A. F.	11	5	34.2	0.963	0.653	+0.310	1.310	1.023	+0.287
E. C.	11	7	30.7	0.964	0.541	+0.423	1.296	0.946	+0.350
L. G.	13	6	54.9	1.017	0.395	+0.622	1.460	0.933	+0.527

TABLE IV.

Calcium and Phosphorus Storage According to Weight, in Grams per Kilo per Day.

Name.	Age.		Weight.	Calories.	Protein.	Calcium.			Phosphorus.		
						In-take.	Out-put.	Balance.	In-take.	Out-put.	Balance.
	yr.	mo.	kg.		gm.	gm.	gm.	gm.	gm.	gm.	gm.
K. C.	3	7	16.2	86	3.1	0.054	0.043	+0.011	0.062	0.054	+0.008
C. B.	3	8	15.2	83	3.3	0.058	0.048	+0.010	0.067	0.056	+0.011
M. O.	4	7	19.5	78	2.8	0.046	0.037	+0.009	0.055	0.047	+0.008
A. B.	6		21.4	84	3.1	0.043	0.033	+0.010	0.058	0.053	+0.005
M. P.	6		19.1	78	2.6	0.039	0.027	+0.012	0.045	0.039	+0.006
R. G.	7	4	25.2	61	2.1	0.035	0.024	+0.011	0.039	0.034	+0.005
R. B.	8	9	39.3	38	1.2	0.017	0.010	+0.007	0.020	0.018	+0.002
R. T.	9	6	33.0	52	1.8	0.027	0.019	+0.008	0.031	0.025	+0.006
L. M.	10	11	35.4	73	2.7	0.028	0.019	+0.009	0.039	0.030	+0.009
A. F.	11	5	34.2	67	2.5	0.028	0.019	+0.009	0.038	0.030	+0.008
E. C.	11	7	30.7	77	2.9	0.031	0.017	+0.014	0.042	0.031	+0.011
L. G.	13	6	54.9	55	2.0	0.018	0.007	+0.011	0.027	0.017	+0.010

TABLE V.

Average Calcium and Phosphorus Storage of Children, in Grams per Kilo per Day.

Age.	No. of cases.	Calcium.			No. of cases.	Phosphorus.			References.
		In-take.	Out-put.	Balance.		In-take.	Out-put.	Balance.	
		gm.	*gm.*	*gm.*		*gm.*	*gm.*	*gm.*	
1–6 mo.	32	0.090	0.063	+0.027	11	0.075	0.058	+0.017	(10–20, 25, 27)
7–12 mo.	14	0.137	0.098	+0.039	5	0.115	0.087	+0.028	(9, 12, 16, 18, 21–23, 25, 28)
1–2 yrs.	5	0.089	0.070	+0.019					(12)
3–8 yrs.	13	0.039	0.028	+0.011	10	0.054	0.046	+0.008	(7, 26, 27, Table IV above)
9–14 yrs.	14	0.027	0.018	+0.009	11	0.039	0.029	+0.010	(7, 8, 24, Table IV above)

Second Series.

This series of experiments was an intensive study of the calcium metabolism of three children to determine on what amount of calcium they made optimum storage. Milk was again used as the chief source of calcium, and the variations of calcium intake were accomplished by regularly graduated alterations in the daily allowance of milk. The series consisted of eight consecutive experiments of 6 days each. During the first experiment each child received 250 gm. of milk per day; during the second, 500 gm.; the third, 750 gm.; the fourth, 1,000 gm.; the fifth, 1,500 gm.; the sixth, 1,000 gm.; the seventh, 750 gm.; the eighth, 500 gm. per day. Each 6 day experiment was divided into two 3 day periods for sampling and analysis of food and collection and analysis of urine and feces. The first 3 days of each experiment were intended to allow for adjustment to the diet and the second 3 day period to show how much storage the diet would induce.

Experience showed, however, that it was at least equally satisfactory to use the average of all 6 days. In addition to milk, the diet used in this series of experiments furnished bread, butter, orange juice, and oatmeal daily with potato and macaroni in alternate 3 day periods. Apple sauce, corn flakes, and prunes were also used. The amount of each food consumed by each child is

TABLE VI.

Average Daily Food Intake During the Second Series of Experiments, in Grams per Day.

Experiment No.	I	II	III	IV	V	VI	VII	VIII
E. C., female, age 12 years; weight 33.4 to 35.4 kilos.								
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Milk	250	500	750	1,000	1,500	1,000	750	500
Bread	288	440	406	382	370	325	381	334
Butter	30	30	30	30	30	30	30	30
Orange juice	60	75	85	69	58	60	60	64
Oatmeal	200	100	167	150	67	150	117	100
Potato	459		400		388		442	
Macaroni		369		394		460		463
Cocoa	180	187						175
Apple sauce	134	275						
Corn flakes			50	47	44	50		
Prunes							100	100
Sugar	45	14	6	8	8	10	12	16
Strawberries							33	3
Cream of wheat		17						
Calories	1,863	2,784	2,357	2,946	2,678	2,936	2,274	2,634
Protein	51	90	77	103	94	101	71	86
Calcium	0.425	0.748	0.994	1.273	1.794	1.262	1.015	0.741
Phosphorus	0.886	1.211	1.460	1.619	2.009	1.559	1.367	1.128
M. O., female, age 5 years, 2 months; weight 20.2 to 22.1 kilos.								
Milk	250	500	750	1,000	1,500	1,000	750	500
Bread	224	303	243	284	182	175	281	277
Butter	28	25	25	25	25	25	25	25
Orange juice	60	58	60	62	58	60	60	64
Oatmeal	150	75	142			100	100	100
Potato	242		150		134		142	
Macaroni		157		188		215		167
Cocoa	117	134						200
Apple sauce	125	150						
Corn flakes			30	30	30	29		
Prunes							64	60
Sugar	35	10	10	5	5	10	11	10
Strawberries							19	
Cream of wheat		67						
Calories	1,469	1,932	1,687	2,112	1,922	1,960	1,753	1,844
Protein	39	61	56	74	71	68	57	60
Calcium	0.386	0.682	0.930	1.208	1.727	1.196	0.954	0.686
Phosphorus	0.697				1.675		1.133	0.950

TABLE VI—*Concluded.*

Experiment No.	I	II	III	IV	V	VI	VII	VIII
K. C., female, age 4 years, 2 months; weight 17.0 to 18.6 kilos.								
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Milk........................	250	500	750	1,000	1,500	1,000	750	500
Bread......................	222	319	235	217	101	161	237	200
Butter.....................	28	25	25	25	23	25	25	25
Orange juice...............	60	58	60	62	58	60	60	64
Oatmeal....................	142	75	150	150	75	84	100	100
Potato.....................	259		200		142		150	
Macaroni...................		117		142		72		142
Cocoa......................	125	134						200
Apple sauce................	100							
Corn flakes................			30	30	24	29		
Prunes.....................							52	52
Sugar......................	38	10	10	10	7	9	12	13
Strawberries...............							19	
Cream of wheat............		67						
Calories....................	1,341	1,812	1,690	1,976	1,761	1,653	1,627	1,608
Protein....................	40	60	56	68	65	57	53	52
Calcium...................	0.386	0.671	0.933	1.206	1.717	1.177	0.942	0.665
Phosphorus................	0.695	0.930	1.167	1.343		1.198		0.863

shown in Table VI. The technique of weighing, sampling, and analysis of intake and output was similar to that described for the first series and three of the same children, aged 4, 5, and 12 years, served as subjects.

Tables VII and VIII show the results of these experiments, averaged both for 3 day and for 6 day periods.

The data show that E. C. (12 years old) stored only 0.007 gm. of calcium per kilo of body weight when receiving 250 gm. of milk per day. As the intake of milk was increased there was increased retention of calcium up to 0.022 gm. per kilo with 1,000 gm. of milk and 0.023 gm. per kilo with 1,500 gm. of milk. Examination of the results obtained with K. C. (age 4 years) shows the same response to increased intake except that the differences in amounts of calcium stored were not so great. The results with M. O. (age 5 years) were less regular and the storage of calcium was lower throughout. The results of the series as a whole indicate 1,000 gm. of milk per day as the most probable intake required to support an optimum storage of calcium in the body.

TABLE VII.

Calcium and Phosphorus Storage on Varying Amounts of Milk (Second Series), in Grams per Day.

Experiment No.	Calcium.				Phosphorus.			
	Intake.	Urine.	Feces.	Balance.	Intake.	Urine.	Feces.	Balance.
E. C., female, age 12 years; weight 33.4 to 35.4 kilos.								
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
I, 1	0.425	0.026	0.204	+0.195	0.893	0.438	0.280	+0.175
2	0.425	0.018	0.158	+0.249	0.879	0.487	0.236	+0.156
II, 1	0.731	0.034	0.240	+0.457	1.124	0.650	0.230	+0.244
2	0.764	0.033	0.277	+0.454	1.297	0.371	0.357	+0.569
III, 1	0.981	0.021	0.230	+0.730	1.398	0.549	0.199	+0.650
2	1.006	0.033	0.245	+0.727	1.522	0.584	0.258	+0.680
IV, 1	1.272	0.087	0.432	+0.753	1.604	0.714	0.304	+0.586
2	1.273	0.102	0.361	+0.810	1.634	0.750	0.272	+0.612
V, 1	1.803	0.119	0.799	+0.885	2.082	1.193	0.442	+0.447
2	1.785	0.127	0.892	+0.766	1.935	1.140	0.329	+0.466
VI, 1	1.265	0.129	0.516	+0.620	1.570	0.943	0.338	+0.289
2	1.259	0.178	0.429	+0.652	1.547	0.881	0.278	+0.388
VII, 1	1.012	0.126	0.438	+0.448	1.352	0.928	0.289	+0.135
2	1.019	0.099	0.281	+0.639	1.382	0.883	0.191	+0.308
VIII, 1	0.746	0.102	0.238	+0.406	1.146	0.536	0.199	+0.411
2	0.735	0.121	0.215	+0.399	1.110	0.529	0.195	+0.386
M. O., female, age 5 years, 2 months; weight 20.2 to 22.1 kilos.								
I, 1	0.381	0.023	0.284	+0.074	0.675	0.334	0.289	+0.052
2	0.392	0.016	0.253	+0.123	0.719	0.325	0.256	+0.138
II, 1	0.679	0.016	0.472	+0.191	0.920	0.369	0.308	+0.243
2	0.684	0.019	0.417	+0.248				
III, 1	0.929	0.025	0.713	+0.191				
2	0.931	0.022	0.704	+0.205				
IV, 1	1.207	0.027	0.905	+0.275				
2	1.209	0.023	0.949	+0.237				
V, 1	1.735	0.033	1.494	+0.208	1.730	0.658	0.769	+0.303
2	1.719	0.021	1.473	+0.225	1.620	0.699	0.737	+0.184
VI, 1	1.196	0.022	0.863	+0.311	1.278	0.578	0.474	+0.226
2	1.195	0.026	0.895	+0.274				
VII, 1	0.952	0.022	0.730	+0.200	1.119	0.523	0.359	+0.237
2	0.955	0.025	0.786	+0.144	1.147	0.529	0.415	+0.203
VIII, 1	0.700	0.034	0.565	+0.101	1.014	0.436	0.321	+0.257
2	0.671	0.025	0.504	+0.142	0.886	0.450	0.261	+0.175

TABLE VII—*Concluded.*

Experiment No.	Calcium.				Phosphorus.			
	Intake.	Urine.	Feces.	Balance.	Intake.	Urine.	Feces.	Balance.

K. C., female, age 4 years, 2 months; weight 17.0 to 18.6 kilos.

Experiment No.	Calcium				Phosphorus			
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
I, 1	0.386	0.019	0.180	+0.187	0.688	0.239	0.168	+0.281
2	0.385	0.010	0.203	+0.172	0.701	0.283	0.180	+0.238
II, 1	0.668	0.027	0.321	+0.320	0.894	0.379	0.185	+0.330
2	0.674	0.029	0.453	+0.192	0.965	0.447	0.249	+0.269
III, 1	0.931	0.037	0.562	+0.332	1.159	0.539	0.266	+0.354
2	0.934	0.031	0.667	+0.236	1.175	0.543	0.282	+0.350
IV, 1	1.210	0.049	0.858	+0.303	1.351	0.780	0.333	+0.238
2	1.202	0.037	0.886	+0.279	1.334	0.669	0.393	+0.272
V, 1	1.731	0.046	1.449	+0.236	1.733	0.759	0.698	+0.276
2	1.702	0.025	1.290	+0.387				
VI, 1	1.174	0.035	0.939	+0.200	1.186	0.606	0.376	+0.204
2	1.179	0.021	0.836	+0.322	1.210	0.553	0.322	+0.335
VII, 1	0.940	0.035	0.592	+0.313	1.077	0.509	0.241	+0.327
2	0.943	0.044	0.678	+0.221				
VIII, 1	0.670	0.034	0.409	+0.227	0.891	0.541	0.190	+0.160
2	0.659	0.034	0.440	+0.185	0.834	0.500	0.210	+0.124

Third Series.

The experiments of this series were undertaken to determine whether children could utilize calcium in the form of vegetables as efficiently as they had utilized the calcium of milk in the experiments described above. The three children who had been used in the second series served again as subjects.

There were three consecutive experiments of 9 days each, this longer time being allowed in order to provide ample opportunity for adjustment to the dietary changes involved. Each child received throughout these experiments 500 gm. of milk per day with bread, butter, oatmeal, and orange juice. During the first 9 days they received a large amount of potato, selected as a familiar and acceptable vegetable of low calcium content. During the second 9 days they received, instead of the potato, carrots and spinach in such amount as to make the total calcium of the diet the same as that of the diet containing 1,000 gm. of milk which in the second series had induced optimun storage. During the last 9 days the diet was the same as during the first. Each of the

day experiments was divided into three 3 day periods for collection and analysis of foods, feces, and urine.

TABLE VIII.

Average Calcium and Phosphorus Balances for the Second Series of Experiments, in Grams per Day.

Experiment No.	Calcium.				Phosphorus.			
	Intake.	Output.	Balance.	Balance per kilo.	Intake.	Output.	Balance.	Balance per kilo.
E. C., female, age 12 years; weight 33.2 to 35.4 kilos.								
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
I	0.425	0.203	+0.222	+0.007	0.886	0.721	+0.165	+0.005
II	0.748	0.292	+0.456	+0.013	1.211	0.804	+0.407	+0.012
III	0.994	0.265	+0.729	+0.021	1.460	0.795	+0.665	+0.019
IV	1.273	0.491	+0.782	+0.022	1.619	1.020	+0.599	+0.017
V	1.794	0.969	+0.825	+0.023	2.009	1.552	+0.457	+0.013
VI	1.262	0.626	+0.636	+0.018	1.559	1.220	+0.339	+0.010
VII	1.015	0.472	+0.544	+0.015	1.367	1.146	+0.221	+0.006
VIII	0.741	0.338	+0.403	+0.011	1.128	0.730	+0.398	+0.011
M. O., female, age 5 years, 2 months; weight 20.2 to 22.1 kilos.								
I	0.386	0.288	+0.098	+0.005	0.697	0.602	+0.095	+0.005
II	0.682	0.462	+0.220	+0.011				
III	0.930	0.732	+0.198	+0.009				
IV	1.208	0.952	+0.256	+0.012				
V	1.727	1.510	+0.217	+0.010	1.675	1.432	+0.243	+0.011
VI	1.196	0.903	+0.293	+0.014				
VII	0.954	0.782	+0.172	+0.008	1.133	0.913	+0.220	+0.010
VIII	0.686	0.564	+0.122	+0.006	0.950	0.734	+0.216	+0.010
K. C., female, age 4 years, 2 months; weight 17.0 to 18.6 kilos.								
I	0.386	0.206	+0.180	+0.011	0.695	0.435	+0.260	+0.015
II	0.671	0.415	+0.256	+0.015	0.930	0.630	+0.300	+0.017
III	0.933	0.649	+0.284	+0.016	1.167	0.815	+0.352	+0.020
IV	1.206	0.915	+0.291	+0.016	1.343	1.088	+0.255	+0.014
V	1.717	1.405	+0.312	+0.017				
VI	1.177	0.916	+0.261	+0.015	1.198	0.929	+0.269	+0.015
VII	0.942	0.675	+0.267	+0.015				
VIII	0.665	0.459	+0.206	+0.011	0.863	0.721	+0.142	+0.008

Tables IX, X, and XI give the detailed data of these experiments presented in the same general manner as in the previous series.

TABLE IX.

Average Daily Food Intake during the Third Series of Experiments, in Grams per Day.

Food.	Experiment I.			Experiment II.			Experiment III.		
	1	2	3	1	2	3	1	2	3
E. C., female, age 12 years, 9 months; weight 36.6 to 38.4 kilos.									
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Milk	500	500	500	500	500	500	500	500	500
Bread	317	474	579	633	593	598	567	500	467
Butter	30	30	30	30	30	30	30	30	30
Orange juice	73	80	80	80	80	80	80	80	80
Oatmeal	150	150	150	150	150	150	150	150	150
Potato	483	577	600				589	600	600
Spinach				279	350	349			
Carrots				279	300	317			
French dressing				16	15	15			
Sugar	10	10	10	15	20	20	15	15	
Calories	1,815	2,272	2,554	2,711	2,650	2,669	2,539	2,370	2,2
Protein	58	74	84	92	91	92	83	77	74
Calcium	0.687	0.740	0.774	1.201	1.267	1.258	0.769	0.750	0.740
Phosphorus	0.945	1.109	1.205	1.349	1.417	1.428	1.191	1.139	1.1
M. O., female, age 5 years, 10 months; weight 22.0 to 23.0 kilos.									
Milk	500	500	500	500	500	500	500	499	500
Bread	245	303	358	387	402	367	333	179	217
Butter	25	25	25	25	25	25	25	25	25
Orange juice	73	80	80	80	80	80	80	80	80
Oatmeal	142	150	150	150	167	167	183	83	150
Potato	417	450	450				450	433	450
Spinach				222	250	250			
Carrots				222	250	283			
French dressing				8	12	12			
Sugar	5	5	5	10	15	15	10	10	10
Calories	1,531	1,707	1,842	1,924	2,037	1,955	1,828	1,353	1,505
Protein	50	56	61	67	70	67	60	42	48
Calcium	0.659	0.679	0.696	1.033	1.071	1.056	0.691	0.639	0.653
Phosphorus	0.860	0.924	0.970	1.079	1.162	1.146	0.961	0.791	0.851
K. C., female, age 4 years, 11 months; weight 18.7 to 19.8 kilos.									
Milk	500	500	500	500	500	500	500	500	500
Bread	180	203	276	275	263	232	250	233	183
Butter	25	25	25	25	25	25	25	25	25
Orange juice	73	80	80	80	80	80	80	80	80

TABLE IX—*Concluded*.

Food.	Experiment I.			Experiment II.			Experiment III.		
	1	2	3	1	2	3	1	2	3

K. C., female, age 4 years, 11 months; weight 18.7 to 19.8 kilos—*Concluded*.

Food	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Oatmeal..............	142	100	117	150	150	183	200	200	200
Potato...............	517	483	450				467	494	517
Spinach..............				208	250	250			
Carrots..............				208	250	267			
French dressing.......				12	15	15			
Sugar................	5	5	5	10	15	15	10	10	10
Calories..............	1,412	1,432	1,615	1,650	1,686	1,632	1,631	1,601	1,482
Protein..............	45	46	53	56	57	55	53	52	47
Calcium..............	0.646	0.646	0.668	0.977	1.028	1.014	0.670	0.667	0.653
Phosphorus...........	0.838	0.833	0.889	0.971	1.040	1.034	0.902	0.897	0.863

The results here are more variable than in either of the other series, but show in the case of each of the children studied a much lower storage of calcium on the high vegetable diet than on the diets previously used in which the same or even smaller amounts of calcium were supplied in the form of milk. The phosphorus balances are discussed in connection with those of the fourth series below.

Fourth Series.

Since the experiments of the third series were made in February and it has been found (29) that in general children do not maintain such active growth in winter as in summer, it was thought that this might perhaps have been a factor in the low rates of storage of calcium and phosphorus on the diets in which vegetables replaced half of the milk. Hence another series of experiments (fourth series) was carried out during August and September. The plan of the experiments was also modified to provide for a more direct comparison of the vegetables and milk as sources of calcium for the growing child. In this (fourth) series, 750 gm. of milk per day were given during the first 9 days; during the next 15 days the daily allowance of milk was reduced to 375 gm. and enough vegetables were added to yield an equivalent amount of calcium;

TABLE X.

Calcium and Phosphorus Balances in the Experiments of the Third Series, in Grams per Day.

Experiment No.		Calcium.				Phosphorus.			
		Intake.	Urine.	Feces.	Balance.	Intake.	Urine.	Feces.	Balance.
E. C., female, age 12 years, 9 months; weight 36.6 to 38.4 kilos.									
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
I,	1	0.687	0.037	0.368	+0.282	0.945	0.557	0.263	+0.125
	2	0.740	0.036	0.550	+0.154	1.109	0.722	0.354	+0.033
	3	0.774	0.047	0.693	+0.034	1.205	0.785	0.430	−0.010
II,	1	1.201	0.065	0.801	+0.335	1.349	0.847	0.313	+0.189
	2	1.267	0.056	1.231	−0.020	1.417	0.924	0.429	+0.064
	3	1.258	0.067	1.048	+0.143	1.428	0.947	0.352	+0.129
III,	1	0.769	0.070	0.583	+0.116	1.191	0.672	0.337	+0.182
	2	0.750	0.068	0.553	+0.129	1.139	0.738	0.356	+0.045
	3	0.740	0.057	0.577	+0.106	1.111	0.592	0.362	+0.157
M. O., female, age 5 years, 10 months; weight 22.0 to 23.0 kilos.									
I,	1	0.659	0.005	0.491	+0.163	0.860	0.471	0.356	+0.033
	2	0.679	0.009	0.551	+0.119	0.924	0.523	0.354	+0.047
	3	0.696	0.010	0.525	+0.161	0.970	0.587	0.346	+0.037
II,	1	1.033	0.013	0.912	+0.108	1.079	0.622	0.413	+0.044
	2	1.071	0.015	1.014	+0.042	1.162	0.639	0.414	+0.109
	3	1.056	0.016	0.962	+0.078	1.146	0.577	0.400	+0.169
III,	1	0.691	0.013	0.579	+0.099	0.961	0.459	0.427	+0.075
	2	0.639	0.010	0.460	+0.169	0.791	0.529	0.267	−0.005
	3	0.653	0.007	0.596	+0.050	0.851	0.549	0.398	−0.096
K. C., female, age 4 years, 11 months; weight 18.7 to 19.8 kilos.									
I,	1	0.646	0.019	0.549	+0.078	0.838	0.483	0.332	+0.023
	2	0.646	0.016	0.502	+0.128	0.833	0.505	0.281	+0.047
	3	0.668	0.022	0.457	+0.189	0.889	0.541	0.247	+0.101
II,	1	0.977	0.016	0.785	+0.176	0.971	0.627	0.307	+0.037
	2	1.028	0.019	0.901	+0.108	1.040	0.625	0.294	+0.121
	3	1.014	0.015	0.977	+0.023	1.034	0.456	0.352	+0.226
III,	1	0.670	0.020	0.472	+0.178	0.902	0.416	0.258	+0.228
	2	0.667	0.019	0.392	+0.256	0.897	0.548	0.220	+0.129
	3	0.653	0.016	0.563	+0.074	0.863	0.510	0.320	+0.033

after which the subjects returned for a period of 6 days to the diet of the first 9 days. Carrots, spinach, and celery served as the vegetable source of calcium. The rest of the diet was similar to that used in the former experiments—bread, butter, orange juice, oatmeal, potato, and apple.

Three children served as subjects: L. M., a girl of 13 years; V.M., a girl 10 years old; and A. M. a boy 6 years old. Table XII shows the food intake of each child and Tables XIII and XIV

TABLE XI.

Average Calcium and Phosphorus Balances in the Third Series of Experiments, in Grams per Day.

Experiment No.	Calcium.				Phosphorus.			
	Intake.	Output.	Balance.	Balance per kilo.	Intake.	Output.	Balance.	Balance per kilo.
E. C., female, age 12 years, 9 months; weight 36.6 to 38.4 kilos.								
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
I	0.734	0.577	+0.157	+0.004	1.086	1.037	+0.049	+0.001
II	1.242	1.090	+0.152	+0.004	1.398	1.271	+0.127	+0.003
III	0.753	0.636	+0.117	+0.003	1.147	1.019	+0.085	+0.002
M. O., female, age 5 years, 10 months; weight 22.0 to 23.0 kilos.								
I	0.678	0.530	+0.148	+0.007	0.918	0.879	+0.039	+0.002
II	1.053	0.977	+0.076	+0.003	1.129	1.022	+0.107	+0.005
III	0.661	0.555	+0.106	+0.005	0.868	0.876	−0.008	±0.000
K. C., female, age 4 years, 11 months; weight 18.7 to 19.8 kilos.								
I	0.654	0.522	+0.132	+0.007	0.853	0.796	+0.057	+0.003
II	1.006	0.904	+0.102	+0.005	1.015	0.887	+0.128	+0.007
III	0.663	0.494	+0.169	+0.009	0.887	0.757	+0.130	+0.007

show the data of intake and output of calcium and phosphorus. The second girl, V. M., did not continue during the final 6 day period of the series.

In all three cases the storage of calcium during the first 9 days approximates the average figure found in the first series of experiments, 0.01 gm. per kilo of body weight per day and in all three cases the balance became much less favorable when half of the milk was replaced by vegetables. In the case of L. M. tonsilitis

TABLE XII.

Average Daily Food Intake during the Fourth Series of Experiments, in Grams per Day.

L. M., female, age 12 years, 9 months; weight 48 kilos.

Food.	Experiment I.			Experiment II.					Experiment III.	
	1	2	3	1	2	3	4	5	1	2
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Milk	750	750	749	375	375	375	375	375	750	750
Bread	405	467	500	500	520	533	400	468	452	500
Butter	40	40	40	38	40	40	40	40	40	40
Orange juice	70	83	85	85	85	85	85	85	85	85
Oatmeal	150	150	150	150	150	150	150	150	150	150
Potato	483	483	500	117	200	200	200	200	550	550
Apple	65	89	98	58	117	133	104	88	191	210
Spinach				300	330	308	263	296		
Carrots				100	100	100	100	100		
String beans				17						
Celery						40	57	50		
Sugar	8	9	35	8	8	8	8	8	8	8
Calories	2,277	2,461	2,662	2,218	2,352	2,405	2,036	2,207	2,512	2,646
Protein	73	79	82	73	76	78	65	72	79	83
Calcium	1.027	1.047	1.057	1.030	1.076	1.091	1.002	1.056	1.053	1.068
Phosphorus	1.285	1.343	1.379	1.078	1.139	1.152	1.019	1.091	1.366	1.406

V. M., female, age 10 years, 3 months; weight 32.4 to 32 kilos.

Food.	Experiment I.			Experiment II.				
	1	2	3	1	2	3	4	5
Milk	750	750	750	375	375	375	375	375
Bread	167	252	300	333	253	247	351	358
Butter	40	40	40	38	37	38	40	40
Orange juice	70	83	85	85	85	85	85	85
Oatmeal	150	150	150	150	150	133	150	150
Potato	450	450	450	117	200	100	100	100
Apple	66	80	92	62	82	66	113	88
Spinach				266	243	196	200	200
Carrots				117	100	167	200	200
String beans				28				
Celery						50	100	100
Sugar	5	6	35	5	5	5	5	5
Calories	1,534	1,872	2,121	1,779	1,587	1,546	1,896	1,899
Protein	50	59	63	57	50	48	59	60
Calcium	0.959	0.985	0.999	0.953	0.874	0.889	0.981	0.981
Phosphorus	1.075	1.152	1.193	0.931	0.858	0.833	0.959	0.962

TABLE XII—*Concluded.*

Food.	Experiment I.			Experiment II.					Experiment III.	
	1	2	3	1	2	3	4	5	1	2
A. M., male, age 6 years, 1 month; weight 17.1 to 17.9 kilos.										
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Milk............	750	750	750	375	375	375	375	375	750	750
Bread...........	108	180	200	200	200	237	227	198	205	200
Butter..........	27	30	30	30	30	30	30	30	30	30
Orange juice.....	70	83	85	85	85	77	85	85	85	85
Oatmeal........	150	150	150	150	150	150	150	150	150	150
Potato..........	400	400	400	117	200	200	200	200	450	450
Apple...........		62	69	53	59	22	55	33	51	26
Spinach.........				233	275	247	250	216		
Carrots.........				133	100	100	100	100		
String beans.....				17						
Celery..........							33	10	46	
Sugar...........	5	5	30	5	5	5	5	5	5	5
Calories.........	1,318	1,571	1,728	1,363	1,390	1,460	1,454	1,364	1,651	1,622
Protein..........	44	51	53	44	45	49	47	44	54	53
Calcium.........	0.932	0.958	0.964	0.876	0.860	0.910	0.897	0.873	0.968	0.965
Phosphorus......	0.999	1.069	1.089	0.803	0.803	0.857	0.850	0.815	1.106	1.099

complicated the last 6 days (but only these) of the second period. V. M. found it so difficult to accept the high vegetable diet that her food intake was not uniform during the first half of the second period. A. M. showed the most favorable results with the high vegetable diet but even here the substitution of vegetables for half of the milk affected the calcium balance very unfavorably.

Rose has shown (30) that it is possible to meet the calcium requirement of the human adult by the use of carrots, and somewhat similar results have been published by Blatherwick and Long (31) since the experiments here described were made. There is no doubt that the calcium of vegetables is assimilable by man and is a factor in the food value of the vegetables and one reason for including them in the diet. The experiments which we here described appear to show quite definitely that milk is much superior to vegetables as a source of calcium for the growing child. The superiority of milk to vegetables as a source of calcium for dogs had previously been shown by McClugage and Mendel (32).

TABLE XIII.

Calcium and Phosphorus Balances for the Fourth Series of Experiments, in Grams per Day.

Experiment No.		Calcium.				Phosphorus.			
		Intake.	Urine.	Feces.	Balance.	Intake.	Urine.	Feces.	Balance.
L. M., female, age 12 years, 9 months; weight 48 kilos.									
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
I,	1	1.027	0.067	0.554	+0.406	1.285	0.768	0.335	+0.182
	2	1.047	0.076	0.498	+0.473	1.343	0.822	0.292	+0.229
	3	1.057	0.082	0.539	+0.436	1.379	0.748	0.299	+0.332
II,	1	1.030	0.066	0.738	+0.226	1.078	0.797	0.305	−0.024
	2	1.076	0.046	0.704	+0.326	1.139	0.860	0.326	−0.047
	3	1.091	0.054	0.808	+0.229	1.152	0.776	0.255	+0.121
	4	1.002	0.046	0.793	+0.163	1.019	0.837	0.331	−0.149
	5	1.056	0.054	0.912	+0.090	1.091	0.861	0.318	−0.088
III,	1	1.053	0.059	0.661	+0.333	1.366	0.897	0.398	+0.069
	2	1.068	0.079	0.618	+0.371	1.408	0.931	0.353	+0.124
V. M., female, age 10 years, 3 months; weight 32.4 to 32.0 kilos.									
I,	1	0.959	0.098	0.595	+0.266	1.075	0.680	0.296	+0.099
	2	0.985	0.122	0.616	+0.247	1.152	0.767	0.221	+0.164
	3	0.999	0.141	0.588	+0.270	1.193	0.802	0.247	+0.144
II,	1	0.953	0.142	0.709	+0.102	0.931	0.763	0.238	−0.070
	2	0.874	0.105	0.611	+0.158	0.858	0.676	0.214	−0.032
	3	0.889	0.104	0.811	−0.026	0.833	0.634	0.240	−0.041
	4	0.981	0.104	0.736	+0.141	0.959	0.737	0.219	+0.003
	5	0.981	0.090	0.691	+0.200	0.962	0.663	0.224	+0.075
A. M., male, age 6 years, 1 month; weight 17.1 to 17.9 kilos.									
I,	1	0.932	0.032	0.669	+0.231	0.999	0.477	0.299	+0.223
	2	0.958	0.031	0.710	+0.217	1.069	0.567	0.298	+0.204
	3	0.964	0.029	0.786	+0.149	1.089	0.620	0.324	+0.145
II,	1	0.876	0.021	0.739	+0.116	0.803	0.555	0.252	−0.004
	2	0.860	0.016	0.694	+0.150	0.803	0.496	0.294	+0.013
	3	0.910	0.015	0.820	+0.075	0.857	0.516	0.281	+0.060
	4	0.897	0.010	0.798	+0.089	0.850	0.526	0.306	+0.022
	5	0.873	0.014	0.767	+0.092	0.815	0.486	0.286	+0.043
III,	1	0.968	0.025	0.642	+0.301	1.106	0.602	0.299	+0.205
	2	0.965	0.025	0.638	+0.302	1.099	0.306	0.344	+0.449

The six children studied by us will be seen to have given somewhat variable results as regards storage of phosphorus on the vegetable-rich diets.   In some cases (third series) they stored as much phosphorus from the vegetable-rich as from the milk-rich diets, while in other cases the phosphorus balance like the calcium balance was very unfavorably affected when vegetables were substituted for half of the milk in the diet.

TABLE XIV.

Average Calcium and Phosphorus Balances for the Fourth Series of Experiments, in Grams per Day.

Experi-ment No.	Calcium.				Phosphorus.			
	Intake.	Output.	Balance.	Balance per kilo.	Intake.	Output.	Balance.	Balance per kilo.
L. M., female, age 12 years, 9 months; weight 48 kilos.								
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
I	1.044	0.605	+0.439	+0.009	1.336	1.088	+0.248	+0.005
II	1.051	0.844	+0.207	+0.004	1.096	1.133	−0.037	−0.001
III	1.060	0.708	+0.352	+0.007	1.387	1.290	+0.097	+0.002
V. M., female, age 10 years, 3 months; weight 32.4 to 32.0 kilos.								
I	0.981	0.720	+0.261	+0.008	1.140	1.004	+0.136	+0.004
II	0.939	0.824	+0.115	+0.004	0.911	0.924	−0.013	±0.000
A. M., male, age 6 years, 1 month; weight 17.1 to 17.9 kilos.								
I	0.951	0.752	+0.199	+0.011	1.052	0.861	+0.191	+0.011
II	0.894	0.789	+0.105	+0.006	0.833	0.806	+0.027	+0.002
III	0.967	0.665	+0.302	+0.017	1.103	0.776	+0.327	+0.018

SUMMARY AND CONCLUSIONS.

The complete balance of intake and output of calcium (and in most cases also of phosphorus) has been determined in four progressive series of experiments including in all 21 children between the ages of 3 and 14 years and covering a total of 417 experimental days in 139 experiments of 3 days each.

On an ordinary mixed diet containing daily 750 gm. of milk and furnishing a total of 0.74 to 1.02 gm. of calcium per day, children 3 to 13 years of age stored 0.15 to 0.62 gm. of calcium per day, the

amount being approximately proportional to the size of the child and averaging 0.01 gm. of calcium per kilo of body weight per day.

When the daily allowance of milk was increased to 1,000 gm. the storage of calcium was increased. The results obtained indicate that optimum storage of calcium is made when the diet contains 1 quart of milk per day for each child. This, with a normal allowance of other foods, will usually mean a daily intake of at least 1 gm. of calcium for the growing child.

Children do not seem to utilize the calcium of vegetables as efficiently as they do that of milk. In the experiments here reported the calcium balances were more variable and always less favorable when vegetables replaced about half of the milk as source of calcium.

In general the conditions influencing the storage of calcium tended to influence that of phosphorus in the same direction; but as all the experiments here described were planned primarily with reference to calcium, the data for phosphorus are not discussed in corresponding detail.

The finding here reported, that children from 3 to 13 years old require an intake of a gram of calcium per day to induce optimum storage of this element has an important bearing upon standards for calcium in family dietaries. It has been found that the average requirement for maintenance is 0.45 gm. of calcium per man per day, and it has been customary to allow a margin of 50 per cent above this actual maintenance requirement thus making an allowance of 0.68 gm. per day as a "dietary standard" for calcium. Since the child of 3 to 13 years, although eating less food than the man, will need more than 0.68 gm. of calcium in his food to support optimum calcium storage and bone and tooth development, it would seem that a higher dietary standard for calcium, perhaps 1 gm. or more per man per day, would be better in all cases in which the group of people to be fed includes any growing children.

In view of the results obtained with different foods as sources of calcium it is desirable also to emphasize the importance of a quart of milk per day for every child, and it would be best to maintain this level of milk intake up to at least the age of 12 to 14 years.

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THE INFLUENCE OF POSITION AND OF TEMPERATURE UPON THE REACTION OF ALIPHATIC AMINO NITROGEN WITH NITROUS ACID.

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The influence of molecular structure on chemical reactivity is illustrated by a comparison of the time required for the α-amino and the ϵ-amino nitrogen of lysine to react with HNO_2. Van Slyke[1] found that while the α-amino groups of the amino-acids yield their nitrogen completely in 4 to 5 minutes when shaken with HNO_2 a half hour is required for the ϵ-amino group to react quantitatively. The velocity of the reaction is markedly influenced by temperature. At 24°C. the ϵ-amino group of lysine yields its nitrogen in 15 minutes and, according to Sure and Hart,[2] the reaction is completed in 5 minutes by raising the temperature to 37°C. Van Slyke's experiments suggest that the reaction between amino nitrogen and HNO_2 is one of the first order. He found that at 19°C. approximately 70 per cent of the ϵ-amino nitrogen is set free in the first 5 minutes and an additional 25 minutes are required in order that the reaction may be completed. Sure and Hart[2] treated lysine with HNO_2 at 1°C. and measured the gas which was given off after shaking for 5 minutes. They found that the yield of nitrogen corresponded to one-half of the nitrogen content of lysine and from this fact they conclude that low temperatures have a retarding effect on the ϵ-amino and not on the α-amino group of lysine. Without giving due consideration to the time factor Sure and Hart conclude that at 1°C. it is possible to render the ϵ-amino group of lysine entirely inactive.

[1] Van Slyke, D. D., *J. Biol. Chem.*, 1911, ix, 199; 1912, xii, 282.
[2] Sure, B., and Hart, E. B., *J. Biol. Chem.*, 1917, xxxi, 527.

The seemingly sweeping conclusions of Sure and Hart indicate a somewhat paradoxical condition in lysine; *viz.*, that lowering of temperature is without influence on the reactivity of the α-amino group while the same factor serves to prevent the reaction between HNO_2 and the ϵ-amino group. It appears to us that the facts are more nearly in accord with the dynamics of chemical reactions if it be assumed that both reactions are influenced by the factor of temperature. Since, however, the velocity of the reaction between HNO_2 and the α-amino nitrogen is large as compared with that of the ϵ-amino group, a lowering of the temperature, while in reality retarding both reactions, seemingly appears to inhibit the latter reaction entirely. Estimation of the amount of amino nitrogen which is given off when lysine is treated with HNO_2 for a period of 5 minutes is an insufficient criterion on which to base the conclusions which are advanced by Sure and Hart.

It appeared to us that a study of the rates of reactions of aliphatic amino nitrogen when in positions relative to the carboxyl group varying from the alpha to the epsilon affords an excellent opportunity for the correlation of molecular structure and chemical reactivity. Incidentally we have also studied the influence of temperature on the time required for the α- and the ϵ-amino group to react quantitatively. This information is of both practical and theoretical interest. If the conclusions of Sure and Hart with respect to the inactivity of the ϵ-amino group are correct, a basis would be afforded on which a method for the estimation of the α- and the ϵ- amino nitrogen in protein products could be developed. Our data, however, do not bear out the conclusions of these experimenters.

The alanine, β-alanine, and δ-amino-n-valeric acid used in the experiments were Kahlbaum products. The lysine picrate was prepared by the Special Chemicals Co., and the δ-amino-n-valeric acid was procured from the Chemical Manufactures Department of the University of Illinois. Estimations of amino nitrogen showed that all of the products were of a high degree of purity. Casein was prepared according to the procedure of Van Slyke and Bosworth[3] except that the treatment with $(NH_4)_2C_2O_4$ was omitted. The solutions of amino-acids were prepared so that a given aliquot would yield nearly exact equivalent amounts of

[3] Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1913, xiv, 203.

nitrogen. In each instance approximately 15.8 mg. of amino nitrogen were contained in each 25 cc. volumetric flask. A volume of 2 cc. was taken for analysis. This contained approximately 1.26 mg. of nitrogen and yielded slightly more than 2 cc. of gas when the reaction was carried to completion. With the exception of lysine each of the 25 cc. amino-acid solutions contained 2 cc. of 0.1 N NaOH. The lysine picrate was brought into solution by the addition of 11 cc. of 0.1 N NaOH and the casein was dissolved with the aid of 100 mg. of Na_2CO_3. All of the amino nitrogen estimations with the exception of those in which the reaction was allowed to proceed below 15°C. were carried out in a room maintained within 0.5°C. of the indicated temperature by means of a thermoregulator, a hot-plate, and an electric fan. The experiments at 8.5°C. and at 4°C. were carried out in an ice chest. The lower temperature was obtained by placing a large cake of ice near the Van Slyke apparatus and by keeping the air constantly in circulation with the aid of an electric fan. A glass door with conveniently located openings permitted the manipulation of the apparatus.

For the purpose of securing comparable results the following technique was followed in estimating amino nitrogen. All solutions and the apparatus were kept at the indicated temperature for some time before carrying out the estimations of nitrogen. For the lower temperatures it was found necessary to keep the glacial acetic acid at a temperature of 12°C. since at temperatures lower than this it freezes. In order to have the reacting mixture at the indicated temperature, acetic acid at 12°C. was allowed to flow into the reaction chamber, $NaNO_2$ was then added, and the mixture was allowed to stand for some time in order that it might come to the desired temperature. Before adding the solution of amino-acid the temperature of the reaction mixture was taken to insure its being at the same temperature as that indicated by the thermometer on the outside of the apparatus. The speed of the motor was regulated so as to give about 300 vibrations per minute to the deaminizing chamber. 0.5 cc. of water was introduced into the chamber to form a protective layer while admitting the amino-acid solution. This prevented the reaction from proceeding to any considerable extent before the entire amount of amino-acid solution was added. The deaminizing chamber was first shaken by hand in order to secure immediate mixing of the solutions and the shaking was then continued by means of the motor.

The estimation of free amino nitrogen in casein at temperatures above 0°C. was carried out according to the procedure outlined above. The following technique was used in carrying out the reaction at 0°C. A

flask containing 100 cc. of 30 per cent NaNO₂ solution and 50 cc. of glacial acetic acid was brought to a temperature of −0.7°C. by immersion in a vessel which contained a mixture of equal parts of ice and 66 per cent H_2SO_4. At this temperature there was but little evolution of gas. Dry casein was added to the mixture and the flask was occasionally shaken

TABLE I.

The Influence of the Position of the NH_2 Group in Certain Amino-Acids upon the Rate of Deamination with HNO_2 at 25°C.

Time.	Amino nitrogen liberated.									
	Alanine.		β-Alanine.		γ-Amino-n-valeric acid.		δ-Amino-n-valeric acid.		ε-Amino-caproic acid.*	
min.	per cent	K†	per cent	K†	per cent	K†	per cent	K†	per cent	K†
1.0	55	—	57	—	20	—	—	—	—	—
2.0	84	0.46‡	—	—	—	—	36	—	—	—
2.5	96	0.70§	86	0.33	—	—	—	—	—	—
3.0	99	0.85§	90	0.32	58	0.14	—	—	58	—
3.5	97	0.50‡	96	0.41	—	—	51	0.08	—	—
4.0	—	—	95	0.31	—	—	—	—	—	—
4.5	—	—	97	0.32	74	0.14	—	—	—	—
5.0	100	—	97	0.27	78	0.14	82	0.18	—	—
6.0			—	—	—	—	78	0.12	—	—
6.5			—	—	84	0.12	—	—	—	—
7.0			100	—	—	—	—	—	—	—
8.0					92	0.14	—	—	88	0.11
8.5					—	—	88	0.11	—	—
9.0					95	0.15	—	—	—	—
9.5					—	—	—	—	—	—
10.0			100	—	100	—	97	0.17	—	—
11.5					—	—	92	0.09	—	—
13.0					100	—	100	—	97	0.11
13.5							—	—	101	—
15.0							—	—	100	—
17.0							100	—	—	—
18.0									100	—

    * The figures given refer to the ε-amino group of lysine picrate.
    † K = Constant for the monomolecular reaction.
    ‡ Based on the first observer's data.
    § Found by the second observer.

during the course of the reaction. At the conclusion of the desired interval of time the contents of the flask were filtered by suction through a hardened filter paper which had previously been moistened with 10 per cent NaCl solution cooled to −2°C. The deaminized casein was washed repeatedly with NaCl solution to remove the HNO_2, care being taken that in all of the

procedures the temperature did not rise above 0°C. The product was successively washed with distilled water, absolute alcohol, and anhydrous ether and dried in air at 60°C. The substance so obtained is a pale, yellow amorphous powder which does not darken on exposure to air.

The experimental results are presented in Tables I to IV. A number of independent estimations with each of the amino-acids used were carried out by each of the writers both as a check and as a measure of the variability inherent in the method. The factor of error reaches its greatest magnitude when the time

TABLE II.

The Influence of Temperature Upon the Reaction of Alanine with HNO_2.

Time.	Amino nitrogen liberated at.			
	23°C.	15°C.	8.5°C.	4.0°C.
min.	*per cent*	*per cent*	*per cent*	*per cent*
1.0	55	41		
2.0	84	82		
2.5	96	—		
3.0	99	88	61	
3.5	97	—	—	
4.0	—	98	—	
5.0	100	100	78	
6.0		100	—	
8.0			97	
10.0			100	
12.0			100	82
15.0				92
18.0				100
21.0				100

The average value for K at 23°C. is 0.48; at 15°C., 0.45; at 8.5°C., 0.22; and at 4°C., 0.12.

interval is short, since the maximum percentage of total amino nitrogen is given off during the first interval of time. Towards the end of the experiment the amount of gas which is given off is so small that it is impossible to determine whether the gas which is measured represents a continuation of the reaction or experimental error. Since it was not possible for us to secure an amino-acid containing only an ϵ-amino group, use was made of lysine. Estimation of the amino nitrogen in this substance includes, at any time, gas which is given off from both the α- and the ϵ-amino

group.   In order to determine the rate of reaction of the "epsilon" group the first interval of time at which an estimation of nitrogen was carried out was so chosen that all of the α-amino nitrogen

TABLE III.

The Influence of Temperature Upon the Reaction of the ε-Amino Group of Lysine Picrate with HNO_2.

Time.	Amino nitrogen liberated at.						
	30°C.	26°C.	23°C.	19°C.	15°C.	8.5°C.	4°C.
min.	per cent	per cent	per cent	per cent	per cent	per cent	per cent
2.0	41						
3.0	67		58				
4.0	89		—				
5.0	—		—		62		
6.0	95	90	—		—		
8.0	100	99	88		—		
10.0	101	100	—	92	77		
11.0		—	—	92	—		
12.0		100	—	—	—		
13.0			97	97	—		
13.5			101	—	—		
15.0			100	100	—		
17.0			—	100	—		
18.0			100		—		
20.0					95		
25.0					100		
30.0					100	88	
34.0						93	
38.0						100	
40.0						100	
45.0							9
48.0							9
50.0							10
55.0							10

The average value for at K 30°C. is 0.29; at 23°C., 0.11; and at 15°C., 0. The data for other temperatures are insufficient for the calculation of value for K.

was set free.   This time was found from the reaction curve of alanine and in applying these data to the "alpha" group of lysine we do not believe that an error of considerable magnitude is introduced by assuming that the rate of reaction of

the α-amino group of lysine is the same as that of alanine. Our results indicate that the rate of reaction of both the α- and the ϵ-amino group is markedly influenced by temperature. The ϵ-amino group of lysine reacts more slowly than the α-amino group and only about 20 per cent of nitrogen gas will be given off by the former when shaken for 5 minutes with HNO_2 at temperatures near 0°C. The curve (Fig. 1) showing the minimum time required for alanine to yield its nitrogen quan-

TABLE IV.

Influence of Temperature Upon the Reaction of Casein with HNO_2.

Time.	Free amino nitrogen liberated at.			
	21°C.	15°C.	0°C.	
min.	*per cent*	*per cent*	*per cent**	*K†*
5.0			21	
10.0			37	0.020
15.0			46	0.017
18.0	83		—	
21.0	95		—	
24.0	98		—	
25.0	—	87	—	
27.0	100	—	—	
29.0	—	98	—	
30.0	100	—	75	0.020
34.0		100	—	
38.0		100	—	
40.0			90	0.026
60.0			100	
120.0			100	

* The difference between the amino nitrogen content of the original untreated casein and the amino nitrogen left in the treated product.
† K = Constant for the monomolecular reaction.

titatively at varying temperatures indicates that within the usual range of room temperature the reaction is complete within a period of 4 minutes. Under similar conditions a quantitative yield of ϵ-amino nitrogen is obtained in a half hour. For practical purposes considerable time may be gained by the use of the temperature curve for the epsilon group of lysine. The curve necessarily includes experimental errors, hence as a factor of safety 2 to 5 minutes, depending on the temperature, more than the time

indicated by the curve should be allowed in the estimation of the
ε-amino group of lysine. Comparison of our time values with
those given by other investigators for several temperatures shows
a satisfactory agreement.

The values for K calculated according to the monomolecular
formula show a fair agreement, sufficient however to establish
the fact that the reaction between aliphatic amino nitrogen and
HNO_2 is of the first order. This is to be expected since the amount
of HNO_2 present is in such great excess of that used up by the
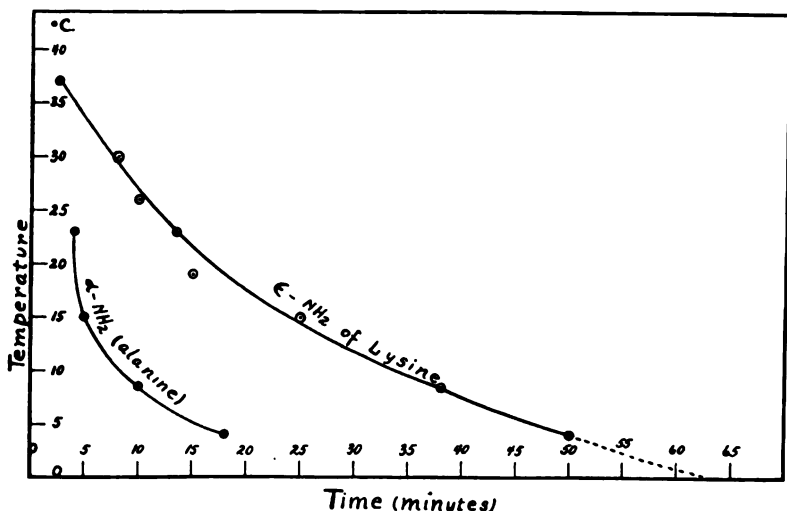
FIG. 1. Curve showing the effect of temperature upon the time required
for the α- and the ε-NH_2 group to react quantitatively with HNO_2.
Point ⊕ taken from the data of Sure and Hart.

reaction that its concentration is practically a constant and the
rate of nitrogen set free is dependent only on the concentration
of amino nitrogen. The probable values of K at 23°C. for the
amino-acids studied are: alanine 0.48, β-alanine 0.33, γ-amino-n-
valeric acid 0.14, δ-amino-n-valeric acid 0.13, ε-amino-caproic
acid 0.11. In studying reactions which proceed with the speed
as indicated by the values for K considerable experimental error
cannot be avoided and this is usually·magnified when the estima-
tions are carried out by several observers.

The results also show that the time required for the amino group of the aliphatic amino-acids which were studied, to yield its nitrogen quantitatively when treated with HNO_2 varies directly as the distance from the carboxyl group. Increasing the distance of the amino group from the carboxyl group necessitates a longer period of time in order that a quantitative yield of nitrogen may be obtained. Fig. 2 also shows the relationship between the position of the amino group of the amino-acids studied and the values of K at 23°C. calculated upon the assumption that the reaction between amino nitrogen and HNO_2 obeys the monomolecular formula. When casein is treated with HNO_2 it will be noted that the time which is required in order to obtain all of the free
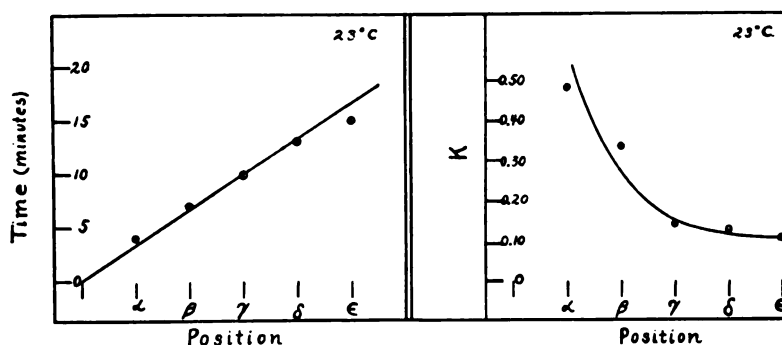
FIG. 2. Curves showing the relation of the position of the amino group (with reference to the COOH group) in certain amino-acids to: (a) the time required to react quantitatively at 23°C. with HNO_2 and; (b) the value of K at 23°C. calculated upon the assumption that the reaction between amino nitrogen and HNO_2 obeys the monomolecular formula.

amino nitrogen is somewhat greater than that necessary for the deamination of the epsilon group of lysine picrate. However, due consideration must be given to the fact that casein in the presence of acetic acid is precipitated from its solution giving a heterogenous mixture. The factor of surface enters. In order to react, the HNO_2 must penetrate the particles of precipitated casein, hence it is to be expected that the reaction time is prolonged. A fairly good agreement between the reaction time of lysine picrate and of casein is obtained by a comparison of the results which were obtained with lysine at 4°C. and with casein at 0°C. The reaction at the low temperature is sufficiently slow so that the factors of surface and penetration are minimized.

SUMMARY.

The rates of deamination of the α- and of the ϵ-amino groups of certain amino-acids are, between 0 and 30°C. markedly influenced by temperature. Contrary to the statement of Sure and Hart, lowering of the temperature was found to decrease the speed of deamination of the α-amino group (of alanine) and not totally to inhibit the complete liberation of nitrogen from the ϵ-amino group of lysine. The influence of the position of the amino group with respect to the carboxyl group in certain other amino-acids upon the time required to yield their nitrogen quantitatively was also studied.

When treated with HNO_2 at ordinary temperatures casein gives off its nitrogen somewhat more slowly than lysine. Reasons for this difference are advanced.

THE WATER-SOLUBLE CONSTITUENTS OF THE ALFALFA PLANT.*

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In a previous paper[1] we described a method whereby relatively large quantities of a chlorophyll-free juice can be obtained from the alfalfa plant within so short a time that autolytic changes presumably are largely avoided. In the present paper we record observations made respecting some of the chemical constituents of this juice.

Recognizing the fact that we are faced by a chemical problem of great complexity, for the present we have refrained from attempting to isolate definite substances from this juice and have directed our efforts to discovering methods whereby fractions containing groups of substances can be obtained which may afford a better starting point for detailed studies than has heretofore been possible.

We have already shown that adding 20 per cent by weight of alcohol to the clear brown freshly expressed chlorophyll-free alfalfa juice precipitates the "colloids" and that these consist chiefly of protein combined with coloring matters, possibly related to the flavones, together with calcium phosphate. This precipitate, which we have called the "colloid precipitate," was equal to about 18 per cent of the solids of the juice of the plants used for this investigation. We have reason to believe that the proportion of the "colloids" varies somewhat with the age of the

[1] Osborne, T. B., Wakeman, A. J., and Leavenworth. C. S., *J. Biol. Chem.*, 1921, xlix, 63.

plant and the conditions of its growth, and for this reason all quantitative data given in this paper apply only to the particular lot of plants examined and, of course, are only approximate.

On long standing the filtrate from the "colloid precipitate" gradually yields a small deposit which, in one case, was equal to about 1.3 per cent of the solids of the juice. This precipitate, which contained about 18 per cent of ash and 11.5 per cent of nitrogen, consisted largely of protein and calcium phosphate, together with "flavone-like" substances, and behaved towards acids and alkalies as does the "colloid precipitate." This appears to be a residue of the latter which was not immediately precipitated by adding 20 per cent of alcohol to the freshly expressed juice. The filtrate from this small spontaneous separation was perfectly clear, bright red in color and free from all colloids, as shown by the absence of any Tyndall effect when exposed to a beam of sunlight. The substances in this solution are, therefore, to be regarded as in true solution.

When this solution was made neutral to litmus a precipitate, consisting chiefly of calcium phosphate and protein, separated. This, when washed with 20 per cent and stronger alcohol and dried at 105°, was equal to 3.4 per cent of the solids of the press-juice. It contained 4.9 per cent of nitrogen and 63.6 per cent of ash. The N × 6.25 was equal to 31 per cent of protein. If all of the nitrogen belongs to protein this, together with the ash, would account for over 94 per cent of this precipitate.

When the alcohol content of the filtrate from the "colloid precipitate" is raised to 53 per cent by weight by adding an equal volume of 93 per cent alcohol a second precipitate, No. II, is produced, which in the experiment to be described was equal to about 14 per cent of the solids of the fresh alfalfa juice used for the following experiments.

. Alfalfa plants from a late cutting (Lot 49) made on Sept. 7, 1921, when the plants were just beginning to blossom, were ground as described in our previous paper,[1] and pressed in the hydraulic press. The first portion of the juice, which was green, was collected separately from the later portion which was brown. The press-cake was ground up with the green juice and pressed again, whereby all of the chlorophyll was retained by the cake. The united brown press-juices measured 4 liters. The press-cakes were washed three times by grinding each time with 4 liters of distilled water and pressing, the final washings being nearly free from color. The results of this procedure are given in the following table.

Alfalfa taken = 7,752 gm. fresh plants, Lot 49.

	Dry solids.*	Nitrogen.	Ash.
	gm.	*gm.*	*gm.*
Taken.........................	1,863	74.22	168.1
Press-juice.......................	469.60	23.50	79.39
1st wash.........................	139.20	5.05	25.60
2nd "	53.34	1.59	9.00
3rd "	23.85	0.76	2.62
Residue..........................	1,105.00	42.72	47.72
Total...........................	1,790.99	73.62	164.33

* All weights of solids were determined by drying for 24 hours or more at 105° to nearly constant weight. It was practically impossible to obtain a really constant weight for most of the fractions obtained, presumably because these are syrups which retain water with great tenacity. The weights given must therefore be regarded as approximate, but we believe they are sufficiently accurate to serve all the purposes for which they can be reasonably employed in interpreting the results of such a preliminary investigation as is described in this paper. In all cases the weights given are corrected for samples previously taken for analysis.

We thus found that of this lot of alfalfa 36.9 per cent of the dry solids, 41.6 per cent of the nitrogen, and 69.3 per cent of the inorganic matter were soluble in water.

About 5 hours after the alfalfa plants had been cut 1,344 cc. of 93 per cent alcohol were added to the 4 liters of brown press-juice, making the alcohol content of the solution about 20.5 per cent by weight. The voluminous precipitate thereby produced was at once collected on two large folded filters and allowed to drain over night. The next morning an equal volume of 93 per cent alcohol was added to the filtrate making its alcoholic content about 53 per cent. This precipitate was filtered out. The precipitate produced by 20 per cent alcohol, which contained the "colloid" constituents of the juice was very voluminous and retained a large amount of the solution from which it had separated, its total solids being equal to 144.57 gm. When washed twice with 20 per cent alcohol and then with stronger alcohol and ether the residue weighed 87.3 gm., equal to 18.6 per cent of the solids of the alfalfa juice. The second alcohol precipitate, which contained 63.31 gm. of solids, was washed with 53 per cent and then with stronger alcohol and ether, 56.4 gm. remaining undissolved, while 6.9 gm. of soluble matter were removed.

The three successive washings of the press-cakes contained 218.4 gm. of solids. If the 1,863 gm. of dry alfalfa contained the "colloids" in the

proportion which the solids of the press-juice bear to those of the washings of the cake the total "colloid" would be 128 gm. or 6.87 per cent of the dry solids of the plant.

Since 57.3 gm. of solids were removed by washing the "colloid precipitate" the 56.4 gm. of the second alcohol precipitate should be greater by the proportion of this 57.3 gm. of solids which would have been precipitated by 53 per cent alcohol if this had been included in the filtrate. Thus 57.3 gm. + 56.4 gm. Precipitate II + 6.5 gm. in washings of Precipitate II + 256.8 gm. in filtrate from Precipitate II = 377.0 gm., of which 57.3 gm. equals 15.2 per cent. This proportion of the 57.3 gm. retained by the colloid precipitate is 8.7 gm., thus making the total corrected amount of Precipitate II = 65.1 gm. or about 13.8 per cent of the solids of the press-juice. The total solids of the press-juice together with those of the water washings were 686 gm., 13.8 per cent of which is 94.7 or 5.1 per cent of the dry alfalfa.

Summarizing the preceding results on the 4 liters of the undiluted press-juice (see p. 412) of the fresh alfalfa plants and including determinations of nitrogen and ash we have the following data.

4 liters of the alfalfa juice (Lot 49) contained:

	Solids.	Nitrogen.	Inorganic.
	gm.	*gm.*	*gm.*
"Colloid precipitate"...............	87.3	9.61	11.59
Precipitate II.....................	65.1	1.86	32.22
Filtrate from Precipitate II........	317.2*	12.05	35.58*
Total.........................	469.6	23.52	79.39

* Calculated by difference.   Directly determined, 312.3 gm. of solids.

We thus find that 40.9 per cent of the nitrogen in the alfalfa juice is precipitated by the addition of 20.5 per cent of alcohol. We have shown in our previous paper[1] that this belongs almost, if not entirely to protein, which, in combination with substances which appear to be similar to flavones, is present in colloidal solution. Precipitate II contains 8 per cent of the original nitrogen, most of which we believe from our examination of this precipitate is likewise protein, and probably largely consists of a small residue of the "colloid" which was not completely precipitated by 20.5 per cent alcohol. Nearly one-half of the nitrogen of the press-juice, therefore, belongs to protein which is almost

completely removed by adding 53 per cent by weight of alcohol.
The alfalfa juice can be thus readily freed from practically all its
protein within a very short time after the growing plants are
cut and the non-protein nitrogenous constituents obtained in an
alcoholic solution so strong that enzymatic, or bacterial changes,
will probably not occur.   Furthermore, by obtaining the press-
juice in the way described substances insoluble in water, but
soluble in alcohol, such as chlorophyll, fat, waxes, and phos-
phatides, do not interfere with a subsequent study of the water-
soluble nitrogenous constituents of the plant.   Such data as we
have obtained respecting these will be given after we have dis-
cussed the distribution of the inorganic constituents among the
three fractions into which the press-juice has been divided.

Distribution of the Inorganic Constituents of the Alfalfa Juice.

Analysis of the ashes of the three fractions just described,
calculated on the basis of 4 liters of the alfalfa juice, gave the
following results:

	"Colloid precipitate."	Precipitate II.	Filtrate.	Total.
	gm.	*gm.*	*gm.*	*gm.*
Ca...............	4.34	8.32	0.65	13.31
Mg...............	0.14	0.53	2.01	2.68
Na...............	0.15	0.00	0.85	1.00
K................	0.02	4.08	15.95	20.05
Fe...............	0.06	Trace.	0.00	0.06
PO_4............	3.84	7.68	1.99	13.51
SO_4............	0.59	10.51	2.23	13.33
SiO_2...........	0.14	0.04	0.16	0.34
CO_2............	2.05	1.48	9.84	13.37
Cl...............	0.00	0.00	2.01	2.01
Total..........	11.33	32.64	35.69	79.66

The close agreement between the sum of these constituents and
the 79.39 gm. calculated from direct determinations of the ash
of the solids of the original juice indicates that these analyses
fairly represent their true composition.

The proportion of each of the above bases and acids pre-
cipitated by 53 per cent alcohol is shown in the following
tabulation.

per cent

Ca.. 95.1

Mg.. 25.0

Na.. 15.0

K.. 20.4

Fe.. 100.0

PO₄.. 85.3

SO₄.. 83.3

SiO₂.. 50.0

CO₃.. 26.4

Cl..None.

   Total ash... 55.1

These figures show that more than one-half of the inorganic constituents are removed by adding 53 per cent of alcohol and that only a small part of the calcium, phosphoric acid, or sulfuric acid, remains in solution. In so far as carbonic acid represents salts of organic acids it appears that about three-fourths of these are not precipitated. This, however, is not an accurate measure of such salts, because the magnesium salts would leave a corresponding quantity of MgO in the ash. Assuming that all of the Mg was thus combined the proportion of CO_3 corresponding thereto plus that found in the ash is only 20 gm. or about 5 per cent of the organic solids of the press-juice.

The PO_4 is nearly all present as inorganic phosphate as shown by the following experiment.

The precipitate produced by making 100 cc. of the filtrate from the "colloid precipitate" alkaline with NaOH was dissolved in dilute nitric acid, precipitated with molybdic solution, and PO_4 determined in the usual way to be 0.1028 gm.

The filtrate from the precipitate produced by neutralizing the original solution gave no turbidity on adding a few drops of $CaCl_2$. This was then evaporated to dryness, ignited, and only 0.0075 gm. of PO_4 found. Whether this latter really represents inorganic phosphorus or calcium phosphate dissolved in the solution of nitrogenous substances with which it is associated has not been determined, but in any event it is clear that at least 95 per cent of the PO_4 is present as PO_4 ions.

The same is true of the SO_4 ions, direct determinations made under different conditions agreeing closely with those made on the ash. It is interesting to note that practically all of the iron is present in the "colloid" precipitate which, when washed with dilute and strong alcohol and dried over H_2SO_4, reacts with both ferro- and ferricyanide.

The magnesium, which is chiefly in the filtrate, is all present in inorganic form as direct determinations made under conditions which should preclude hydrolysis of organic compounds gave the same result as those made on the ash.

Thus to 100 cc. of the filtrate from the "colloid precipitate" $(NH_4)_2C_2O_4$ was added and the calcium oxalate removed by centrifuging. To the clear solution NH_4OH and Na_2HPO_4 were added and after stirring and standing over night the precipitate was removed by centrifuging, dissolved in dilute HCl, and reprecipitated as $MgNH_4PO_4$, equal to 0.0429 gm. of Mg. This agrees exactly with the Mg found in the ash from 100 cc. of the same solution.

The Nitrogen of the Alfalfa Juice.

Although the nitrogenous constituents of the alfalfa juice undergo continuous changes during the growth of the plant and can therefore not be dealt with from a quantitative, or even a qualitative standpoint, with any high degree of accuracy, nevertheless, we found that it is possible to obtain analytical results which should contribute ultimately to a better knowledge of these substances than we now have.

The agricultural chemist formerly regarded the water-soluble non-protein nitrogen as belonging chiefly to amides and it was customary in stating the results of analyses of green fodders to designate this as "amide nitrogen." Later, when it became known that amino-acids were the chief products of protein hydrolysis it seems to have become customary to believe that a large part of this nitrogen belongs to amino-acids, or their peptides, although satisfactory chemical evidence of this, so far as we can find, has never been presented.[2] The filtrate from Precipitate II, which contains one-half of the nitrogen of the alfalfa juice presents an opportunity for attacking this problem under more favorable conditions than those heretofore encountered, because practically all of the protein and other colloidal substances, as well as a considerable part of the inorganic constituents have been removed. In the following pages we record the results of preliminary studies of the various types of nitrogen in this solution.

[2] For a discussion of the literature see Hart, E. B., Humphrey, G. C., and Morrison, F. B., J. Biol. Chem., 1912–13, xiii, 133.

The 4 liters of juice of the alfalfa plants (Lot 49), a part of which was used for the analyses of the inorganic constituents just described, contained 23.5 gm. of nitrogen.   Of this the colloid precipitate contained 9.61 gm. and Precipitate II 1.90 gm., in all 49.5 per cent of the total nitrogen of the juice. As already stated, nearly all of this nitrogen appears to belong to protein, although as yet our evidence is not entirely convincing that a small part may not belong to non-protein substances insoluble in 53 per cent alcohol. The 50.3 per cent of the nitrogen in the filtrate from Precipitate II is equal to 16.1 per cent of the total nitrogen in this lot of alfalfa.

In 100 cc. of this filtrate, which contained 0.120 gm. of total nitrogen and 53 per cent of alcohol, *ammonia* was determined by adding about 300 cc. of water and a slight excess of MgO and then distilling to a small volume. Ammonia equal to 5.75 per cent of the total nitrogen was found in the distillate.   This is equivalent to 2.51 per cent of the total nitrogen in the alfalfa juice, which contained 31.7 per cent of the total nitrogen in the alfalfa.   Another determination was made by evaporating 150 cc. of this filtrate on the steam bath, dissolving the residue in 400 cc. of water, and distilling with MgO as before.   In this case 5.6 per cent of the nitrogen was found as ammonia.

Unless this solution contains extremely unstable nitrogenous compounds it thus appears that practically all of the ammonia was present as ammonium salts.

When the concentrated solutions, from which the ammonia had been removed, were centrifuged, a relatively large orange-yellow deposit was obtained.   When this was washed with water, treated with strong HCl, and the solution shaken with butyl alcohol the aqueous layer was light yellow and the butyl alcohol layer deep ruby-red.

The MgO had, therefore, precipitated a considerable quantity of the coloring matter.

Basic nitrogen was determined by concentrating the filtrate and washings from the MgO precipitate to 100 cc., adding 5 per cent by weight of H_2SO_4 and then a 20 per cent solution of phosphotungstic acid in 5 per cent H_2SO_4 as long as a precipitate formed.   This required about 30 cc. of this reagent. The precipitate was washed with 2.5 per cent phosphotungstic acid in 5 per cent H_2SO_4 solution and the nitrogen determined in it to be equal to 44.4 per cent of the total nitrogen in the filtrate from Precipitate II.   ·

Two other portions of 150 cc. each of the filtrate from Precipitate II, containing 0.1800 gm. of N, were acidified with HCl, evaporated on the steam bath, the residue was dissolved in 75 cc. of 20 per cent HCl and boiled under a reflux for 14 hours.   After evaporating the solutions to dryness the residues were dissolved in 400 cc. of water, and distilled with an excess of MgO.

The ammonia found in the distillate, obtained by boiling the solutions to small volume, in each case was equal to 17.0 per cent of the total nitrogen in the filtrate from Precipitate II, or to about three times as much as before hydrolysis.

After distilling off the ammonia the residue, which was removed by centrifuging, was black and contained a relatively large proportion of humin, presumably derived chiefly from carbohydrates. Nitrogen equal to 11.2 per cent of the nitrogen in the filtrate from Precipitate II was found in the "humin" from one of these determinations. The "humin" from the other determination was treated with strong HCl and shaken with butyl alcohol. The aqueous layer was only slightly colored, but the butyl alcohol layer after centrifuging had a very deep ruby-red color, while the humin formed a coherent mass at the bottom of the butyl alcohol. This layer was washed twice with water and nitrogen determined in both layers. The aqueous solution contained nitrogen equal to 5.4 per cent of the total nitrogen in the filtrate from Precipitate II; the butyl alcohol layer, including the humin, contained 7.8 per cent.

The distribution of nitrogen was similarly determined in the juice of another lot (No. 54) of alfalfa with results as stated below together with those just described for Lot 49.

Since the 53 per cent alcohol solutions used for these determinations had been kept for several months similar analyses were made on a corresponding solution which had been kept for only 3 or 4 days, No. 57.

The distribution of nitrogen both before and after hydrolysis is given in the table below in percentage of the nitrogen in the filtrate from Precipitate II.

	No. 49	No. 54	No. 57
Before hydrolysis.			
Ammonia nitrogen...........................	5.7	3.0	4.5
"Basic nitrogen"...........................	44.4	38.7	51.3
Nitrogen in the MgO precipitate............	1.3		1.4
Free amino nitrogen.......................	29.2	25.2	23.8
After hydrolysis.			
Ammonia nitrogen...........................	17.0	16.9	14.2
"Basic nitrogen"...........................	20.4	25.9	19.0
Nitrogen in the MgO precipitate............	11.2	6.9	16.4
Free amino nitrogen.......................	41.7	46.3	38.3

The fact that a similar small percentage of the nitrogen was obtained as ammonia from the freshly made unhydrolyzed solution from Lot 57 as from those from Lots 49 and 54 which had been kept 8 months indicates that little, if any, change takes place in the nitrogenous constituents when kept in the 53 per cent alcohol solution.

Although these results were obtained with different lots of alfalfa by methods devised for dealing with the products of hydrolysis of proteins the agreement between the analysis is even closer than might be expected. It is evident that the proportion of ammonia nitrogen is greatly increased by hydrolysis while that of the "basic nitrogen" is greatly decreased. Before hydrolysis about one-fourth of the nitrogen reacts with nitrous acid as does free amino nitrogen, after hydrolysis about 40 per cent reacts in this way.

The basic substances from the *unhydrolyzed* filtrate from Precipitate II were examined for arginine, histidine, and lysine by Kossel's method. Although about 8 per cent of the nitrogen in the filtrate from Precipitate II was precipitated by silver nitrate and baryta from the solution obtained by decomposing the phosphotungstic acid precipitate no evidence of the presence of arginine or lysine was obtained. The color reaction for histidine with diazobenzenesulfonic acid as well as the precipitate which formed on adding $HgSO_4$ indicated the presence of this amino-acid, but both of these reactions are given by other nitrogenous substances and consequently cannot be accepted as proof that histidine was actually present.

About 20 liters of the filtrate from Lot 54 were concentrated *in vacuo* at a low temperature to 1,500 cc. This concentrated solution contained 352 gm. of solids, 22.56 gm. of N, and yielded 53.33 gm. of ash, containing 25.6 per cent of CO_2.

Part of this solution, containing 7.9 gm. of solids and 0.509 gm. of N, was diluted to 300 cc. and 5 per cent H_2SO_4 added. Phosphotungstic acid (20 per cent solution in 5 per cent H_2SO_4) was added as long as a precipitate was produced. This was washed with dilute phosphotungstic acid solution and decomposed at room temperature by an excess of baryta. The filtrate and washings from the insoluble barium salts were treated with CO_2, concentrated to small volume, the $BaCO_3$ was washed with water, and the filtrate and washings were made up to 250 cc. Analyses of aliquots of this solution showed it to contain

		N in filtrate from Precipitate II.
	gm.	*per cent*
Total basic nitrogen............................	0.1150	22.6
Amino nitrogen.................................	0.0155	3.0
N in second basic N precipitate................	0.0920	18.1

The total basic nitrogen is equal to only 22.6 per cent of the total nitrogen contained in the filtrate from Precipitate II as against 38.7 per cent found in the phosphotungstic acid precipitate in another portion of this same solution and 44.4 per cent in the corresponding precipitate from Lot 49, previously described. These facts indicate that in decomposing the phosphotungstates with baryta nearly one-half of the basic nitrogen was removed with the baryta precipitates. As this possibility was not foreseen nitrogen was not determined in these precipitates.

The free amino nitrogen is equal to only 13.5 per cent of the total basic nitrogen in the filtrate from the barium salts, a proportion much smaller than should have been found if this basic nitrogen were yielded by arginine, histidine, or lysine.

That arginine, or other substances which yield ammonia on boiling with alkalies, was absent is shown by the fact that when 25 cc. of the 250 cc. of the above solution were diluted with about 400 cc. of 1 per cent NaOH solution and distilled to small volume no ammonia was found in the distillate.

When another portion of 50 cc. of this solution was precipitated by phosphotungstic acid in the usual way 80 per cent of its basic nitrogen was found in the precipitate. In view of the known solubility of most phosphotungstates it appears that practically all of the nitrogen in the 250 cc. of this solution belongs to strongly basic substances.

The remaining 115 cc. of this solution was boiled with an equal volume of concentrated HCl for 7 hours and found to contain 0.0820 gm. of basic nitrogen and 0.0380 gm. of free amino nitrogen, calculated to the original 250 cc. of the solution analyzed. The basic nitrogen thus found after hydrolysis was equal to 71 per cent of the total basic nitrogen as against 80 per cent similarly found before hydrolysis, while the free amino nitrogen had increased from 13.5 to 33.0 per cent of the basic nitrogen. It will require a repetition of this experiment on a scale sufficiently large to permit of the actual isolation of the basic substances before these results can be interpreted.

In another portion of the concentrated filtrate from Precipitate II (Lot 54), containing 0.3008 gm. of N, ammonia was determined by distilling with MgO to be equal to 2.8 per cent of its total nitrogen. After

removing the MgO precipitate and washing this with water, the solution and washings were concentrated *in vacuo* to 200 cc. Of this, 170 cc. were acidified with 5 per cent of H_2SO_4 and precipitated with phosphotungstic acid as described in the preceding experiments. The washed phosphotungstates were decomposed with baryta, the excess of baryta was removed as $BaCO_3$, and the filtrate concentrated. This solution, which reacted strongly alkaline to litmus, was acidified with nitric acid and then treated alternately with silver nitrate and baryta until an excess of silver had been added. The silver precipitate was then decomposed with H_2S in dilute H_2SO_4 solution and the filtrate and washings from the Ag_2S were concentrated to 100 cc. and nitrogen was determined in 10 cc. to be equal to 0.0230 gm. in the whole, or to only 7.7 per cent of the nitrogen in the filtrate from precipitate II. As we have already given evidence of the absence of arginine in the phosphotungstic acid precipitate this nitrogen may represent histidine. Qualitative tests for histidine with diazobenzenesulfonic acid and also with $HgSO_4$ solution gave positive results, but these cannot be accepted as final until this amino-acid is actually isolated.

The filtrate from the silver nitrate-baryta precipitate was freed from silver and baryta by H_2SO_4 and H_2S, made up to 200 cc., and 5 per cent H_2SO_4 added. Phosphotungstic acid was added to this solution as long as a precipitate was produced which was then washed and decomposed with baryta. After removing the excess of barium with carbonic acid the filtrate was concentrated and treated with picric acid under the conditions employed by Kossel for the precipitation of lysine. A relatively large precipitate separated which, when recrystallized, separated in needles somewhat resembling lysine picrate, but which proved to be potassium picrate.[3] The mother liquor when concentrated to a very small volume and allowed to evaporate to dryness left a residue which crystallized in plates. These crystals were insoluble in ether, sparingly soluble in absolute alcohol, but readily soluble in 50 per cent alcohol. No lysine picrate could be isolated.

It is evident that most of the basic nitrogen obtained from the filtrate from Precipitate II belongs to nitrogenous substances quite different from those yielded by protein hydrolysis.

The Coloring Matters of Alfalfa Juice.

The freshly expressed juice of the alfalfa plant is strongly colored and its color for the most part remains in solution after

[3] Potassium phosphotungstate is so insoluble that in working with solutions rich in potassium salts a relatively large proportion may be precipitated with the nitrogenous bases. Since potassium picrate can be easily mistaken for lysine picrate there is danger of a serious explosion if its melting point is taken.

removing the "colloid precipitate" as well as that produced by the addition of 53 per cent alcohol. The concentrated filtrate from this latter precipitate when viewed by reflected light is black, but by transmitted light a clear ruby-red.

Much, if not all, of this color is due to the presence of substances which may belong to the group of flavones, but we want it to be distinctly understood, that as yet we have *no chemical* evidence sufficient to support this view. A part of this color can be removed from the concentrated filtrate by shaking with isoamyl alcohol and the aqueous layer is thus rendered much lighter in color, hence we conclude that a part of the color is in the free state.

After acidifying the aqueous layer and again shaking with isoamyl alcohol a somewhat larger quantity is removed, indicating that acids liberate this coloring matter from salt-like combination.

On boiling the acidified aqueous layer under a reflux condenser a highly hydrated, light brownish product separates which somewhat resembles coagulated protein, but is nearly all soluble in absolute ethyl alcohol with a characteristic deep brown color. In how far this is chemically similar to the products extracted by isoamyl alcohol remains for further investigation.

After removing this insoluble product the aqueous solution is darker than before boiling with acids and contains a relatively large amount of substance soluble in isoamyl alcohol with the same characteristic color as that of the fractions previously extracted by this reagent. In view of the fact that flavones are usually obtained from vegetable sources as glucosides it is probable that the last fraction of the coloring matter was liberated by hydrolysis of some complex of still unknown nature. We have not as yet been able to determine the relative proportions of these four fractions of the coloring matter because none have been obtained in a state of probable purity, nor were any of them free from nitrogen. For this reason we are not prepared to state the proportion in which these highly colored substances occur in the alfalfa juice, but the data which follow show that they unquestionably constitute an important part of the solid matters of the alfalfa juice, and those given in our former paper[1] that they likewise form a considerable part of the insoluble solids of the plant

For the most part these flavone-like substances are combined with the protein and other constituents of the plant from which they are set free by hydrolysis.

That these are widely distributed constituents of vegetable cells and have a physiological importance hitherto unappreciated is indicated by the fact that when colorless yeast cells are hydro-lyzed by boiling with HCl and the hydrolysate is shaken with isoamyl alcohol, the latter separates from the aqueous solution with a deep red color not to be distinguished by the eye from that similarly obtained from the alfalfa. This latter fact also indicates that these colored substances are not especially con-cerned in photosynthetic processes for such do not occur in the yeast cell. These substances deserve further careful study.

The experimental work on which the above statements are founded is described in the following pages.

When about 1.3 per cent of HCl is added to the filtrate from the colloid precipitate a small precipitate is produced which contains both protein and highly colored substances soluble in absolute alcohol, the behavior of which suggests flavone derivatives. This precipitate, which is equal to about 3.5 per cent of the solids of the juice contains nitrogen equivalent to one-third of its weight of protein. The coloring matter could be largely extracted from the precipitate by alcohol containing a very little HCl which suggests that a part of it may be united with the protein in salt-like combination. It is also possible that some of this colored substance was present in the juice as a salt of some inorganic base and that when liberated forms an insoluble compound with the protein.

When the filtrate from the above precipitate is heated on the steam bath for a few hours a voluminous, buff-colored precipitate gradually separates in clumps and skins on the surface of the solution. When this product is removed by centrifuging it forms a dense, dark brown deposit which is almost completely soluble in absolute alcohol. If the solution is boiled with acid stronger than 1.3 per cent, *e.g.* 5 per cent, the amount insoluble in absolute alcohol is greater. This insoluble part consists of a dense black substance resembling humus, which may have originated from carbohy-drates.

The solution of the coloring matter in absolute alcohol is very deep ruby-red. On evaporation it leaves a deep brown residue, readily soluble again in absolute alcohol, or in dilute aqueous NaOH solution. This latter solution appears black by reflected light, or deep yellow by light trans-mitted through thin layers, and is canary-yellow when highly diluted. The alkaline solution yields a chocolate brown flocculent precipitate when neutralized. This precipitate is slightly soluble in water and, when freed from soluble salts, readily passes into a colloidal solution.

No preparation of this product of hydrolysis has yet been obtained free from nitrogen and no method has yet been discovered whereby it can be purified. For this reason we are not yet prepared to venture an opinion respecting its chemical nature, but as we are already in possession of relatively large quantities of material we hope to be able to obtain some evidence of value in the near future.

This substance, which is obviously a product of hydrolysis, of some complex present in the alfalfa, forms about 3 per cent of the solids of the juice. The total amount of the flavone-like substance which is precipitated by acid together with that which subsequently gradually separates on heating is equal to about 5 per cent of the solids of the juice. The filtrate from these precipitates has a deep ruby-red color and still contains much coloring matter.

By repeatedly shaking this solution with isoamyl alcohol a large part of the color is extracted, but at the same time a relatively large amount of hydrochlorides of nitrogenous substances pass into the amyl alcohol. Thus by thrice extracting with isoamyl alcohol and separately evaporating the three successive extracts to small volume *in vacuo* and then repeatedly shaking each with water it was found that the amyl alcohol extracts contained respectively, 2.68, 1.92, and 3.47 per cent of nitrogen, while at the same time a part of the coloring matter had again passed into water used for washing them. Although the nitrogen content of the solids originally extracted by amyl alcohol was reduced to less than one-third, by washing with water it was not practicable to effect a satisfactory separation of its nitrogenous constituents in this way.

The fact that the three fractions above mentioned were together equal to over 10 per cent of the solids of the juice indicates that the colored products of hydrolysis of the water-soluble constituents of the alfalfa juice, even after removing those which separate as solids on boiling with acid, are present in relatively large amount. In view of the fact that flavones unite with both acids or alkalies to form products soluble in water, it is to be expected that a sharp separation of these from hydrochlorides of amino-acids and probably other nitrogenous bases could not be effected in the above described manner. Although we have tried many experiments to make this separation more effective none has, as yet, proved successful.

Shaking with amyl alcohol does, however, effect a good separa-
tion from the inorganic constituents of the juice, and we believe
also from a large part of the non-nitrogenous substances. We
hope, therefore, that ultimately this method may prove helpful
in obtaining fractions from which not only coloring substances,
but also some of the nitrogenous constituents can be obtained in
definite form.   Other immiscible solvents such as ether, benzene,
toluene, petroleum ether, ethyl acetate, or chloroform remove
almost nothing from this solution.   Butyl alcohol on the other
hand extracts the coloring matter readily and, at the same time,
also a large part of the nitrogenous substances.   Further experi-
ence with this solvent may lead to better results than did isoamyl
alcohol, but with either solvent some way must be found to sepa-
rate the coloring matters from the nitrogenous hydrochlorides.

In one experiment the filtrate from the precipitate produced by 53 per
cent alcohol, containing 300 gm. of solids, was concentrated to 1 liter and
shaken with amyl alcohol. When the extract was washed with water
only 6.8 gm. of solids, containing 4.5 per cent of nitrogen and 0.4 per cent
of ash, were found to have been extracted. This shows that only a small
part of the flavone-like substances, which are soluble in amyl alcohol, was
present in the free state. By acidifying the water-soluble part (1,225 cc.)
with 2.5 per cent of hydrochloric acid and again shaking with amyl alcohol
and washing the extract with water after it had been concentrated *in vacuo*,
7.1 gm. of solids containing 2 per cent of nitrogen remained in the amyl
alcohol, indicating that a further part of the coloring matter was liberated
from salt-like combination by the acid. When the water-soluble part was
concentrated *in vacuo* and boiled for 5½ hours at such an acid reaction as was
caused by the addition of 2.5 per cent of HCl, 10.5 gm. of solids, containing
3.0 per cent of nitrogen and 0.3 per cent of ash, separated as a voluminous
precipitate. More than one-half of this fraction was soluble in absolute
ethyl alcohol, the solution being almost black in color. On again shaking
the filtrate with amyl alcohol, and washing the extract with water, 10.5 gm.
of solids, containing 1.9 per cent of nitrogen, were obtained, indicating that
hydrolysis liberated a further quantity of the coloring matter.

When this solution was concentrated *in vacuo* and more HCl added, equal
to 7.2 per cent, 2.0 gm. more of solids were obtained from the amyl alcohol
extract, after this had been washed with water. The aqueous solution was
then boiled for four successive periods of 7 hours, and filtered after each.
The insoluble matter which separated was washed with water and alcohol.
The black humus-like residue weighed 12.4 gm. and contained 4.3 per cent
of nitrogen. This fraction which was insoluble in alcohol was probably
chiefly humin derived from carbohydrates. The dark-colored alcoholic
washings of this humin weighed 2.87 gm. and contained 4.1 per cent of

nitrogen, showing that a little more of the coloring matter had separated on further hydrolysis.

Although 73.3 gm. of HCl had been added up to this point nevertheless when the filtrate from the insoluble hydrolysis products was concentrated to a syrup the acidity of the distillate, as estimated by titration of aliquots, was equal to only 9.2 gm. of HCl and the acidity of this was the same to methyl red as to phenolphthalein.

From this we conclude the solids in this filtrate include a relatively large proportion of strongly basic substances and that notable quantities of volatile organic acids are absent.

The solution was then extracted eleven successive times with 200 cc. of butyl alcohol and the united extracts were concentrated and washed ten times with water. There then remained in the butyl alcohol 19.44 gm. of solids containing 6.0 per cent of nitrogen. The first two water washings were returned to the main solution and the remaining eight concentrated and found to contain 6.2 gm., containing 7.9 per cent, of nitrogen.

The main aqueous solution was then concentrated and HCl added in quantity sufficient, with that already present, to make 15 per cent in all. This solution was then shaken twice with amyl alcohol and each of the extracts was washed with water and solids equal to 9.61 gm., containing 6.0 per cent of nitrogen, were found to have been extracted. Only 1.5 gm. of solids, containing 8.2 per cent of nitrogen, were removed by washing with water, indicating that the greater part of the substance extracted by amyl alcohol is much more readily soluble therein than in water.

The main solution was next extracted 31 times using 400 cc. of normal butyl alcohol each time. Since normal butyl alcohol dissolves a notable quantity of water the volume of the aqueous solution was maintained at about 1,000 cc. by additions of water. The united butyl alcohol extracts were concentrated in vacuo to about 300 cc., the solids which separated were removed by centrifuging and washed with butyl alcohol. These weighed 8.6 gm. and contained 0.608 gm. of N and 6.08 gm. of ash. The fixed ash was equal to 71 per cent and the nitrogen to 24.2 per cent of the volatile part. Since distillation with MgO converted over 93 per cent of this nitrogen into ammonia it is evident that this fraction consisted of inorganic and ammonium salts. The butyl alcohol solution when made up to 500 cc. contained 103.5 gm. of solids, 6.23 gm. of N, and 2.51 gm. of ash, or one-third of both the solids and the nitrogen of the filtrate from the 53 per cent alcohol, but only 5.5 per cent of the ash. This butyl alcohol solution was next washed 26 times with water, 50 cc. being used each time. There were thus removed 82.1 gm. of solids, 6.9 gm. of N, and 2.3 gm. of ash. The butyl alcohol solution was made up to 500 cc. When 5 cc. of this were evaporated in the steam bath and the residue was dried over night at 105° it weighed 0.1535 gm. This, however, on further heating gradually lost weight so that after 24 hours it weighed 0.0933 gm. and after 10 days continuously at 105° weighed only 0.0787 gm. Since we do not know the nature

of the solids in this solution it is at present impossible to determine the cause of this continued loss of weight, nor the amount of solids in this solution or their percentage content of nitrogen. If, as is possible, the butyl alcohol solution contained hydrochlorides of amino-acids these might be converted into butyl esters which would probably be slowly volatile at 105°.

Although the amount of total solids in this solution is thus uncertain it is evident that even taking the highest figure, 15.35 gm., by far the greater part of the solids extracted by normal butyl alcohol again passed into aqueous solution when the butyl alcohol extracts were repeatedly shaken with water.

The main solution which had been extracted 31 times with butyl alcohol as just described, contained 98.5 gm. of solids, 5.14 gm. of N, and 38.1 gm. of ash.

Thus about one-third of the solids, one-fourth of the nitrogen, and more than four-fifths of the inorganic constituents still remained in solution after these repeated extractions, first with isoamyl alcohol and then with butyl alcohol.

Of the total nitrogen left in this solution 1.347 gm. were obtained as ammonia on distilling with MgO and 2.13 as amino nitrogen by the Van Slyke method. If, as is practically certain, the ammonia nitrogen was present as NH_4Cl this would be equal to 5.14 gm. By the conventional method of determining sugar by Fehling's solution an equivalent of 18.5 gm. of dextrose was found. In view of our complete ignorance of the nature of the organic constituents of this solution we have no evidence whatever that the reduction of the cupric oxide was caused by any kind of carbohydrate whatever. Making the purely arbitrary assumption, however, that dextrose was present in the above amount we have calculated the nitrogen content of the remaining organic matter as follows. Deducting the sum of the ash, NH_4Cl, and dextrose from the total solids found there would remain 36.76 gm. of organic solids. Further, deducting the nitrogen as ammonia from the total nitrogen, there remains 3.79 gm. of which the amino nitrogen is equal to 53 per cent. The total nitrogen, other than ammonia, is 10.3 per cent of the above corrected solids.

The solution, containing the 82.1 gm. of solids which had been removed by washing the butyl alcohol extracts with water, was examined with the following results.

	gm.	
Total solids	82.1	
Ash	4.43	= 5.4 per cent of the solids.
Nitrogen	6.88	
N as ammonia	0.571	= 8.3 per cent of the nitrogen.
Amino nitrogen	3.323	= 48.3 per cent " " "
Total chlorine	13.26	= 13.63 gm. of HCl.

This solution contained 13.26 gm. of chlorine as determined by adding an excess of Na_2CO_3, evaporating to dryness, and finding chlorine in the ash. As the ash of the untreated substance contained only a slight trace of chlorine we can assume that this fraction of the solids of the alfalfa juice contained 13.63 gm. of HCl.

When 50 cc. of this solution were concentrated *in vacuo* until a solid transparent residue remained, the distillate contained only 0.1140 gm. of HCl equal to 0.684 gm. from the entire 300 cc.

Hence 95 per cent of the HCl was retained by the solids of this solution thereby showing the presence of a large proportion of strongly basic organic substances.

Assuming that the 0.571 gm. of N found as ammonia was present in ammonium salts this would equal 0.734 gm. of NH_4. If HCl + NH_3 + ash = 18.8 gm. is deducted from the 82.1 gm. of solids we have 63.3 gm. of organic solids containing 6.31 gm. of nitrogen, after deducting that found as ammonia.

The organic solids, therefore, contain 10 per cent of nitrogen, of which 52.7 per cent is free amino nitrogen.

It thus appears that by washing the butyl alcohol extract with water one-third of the total nitrogen of the hydrolyzed solids of the alfalfa can be extracted from the butyl alcohol, chiefly, if not wholly, as hydrochlorides of basic substances. Although this solution contains substances which have a strong affinity for HCl only 23.7 per cent of its total nitrogen was precipitated by phosphotungstic acid under the conditions employed in this laboratory for determining basic nitrogen in the products of protein hydrolysis.

The nature of the nitrogenous substances thus extracted is at present being investigated.

PHOSPHORIC ESTERS OF SOME SUBSTITUTED GLUCOSES AND THEIR RATE OF HYDROLYSIS.

BY P. A. LEVENE AND G. M. MEYER.

WITH THE ASSISTANCE OF I. WEBER.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

A previous communication contained a report[1] on the constants of hydrolysis of several phosphoric acid esters of glucose. The allocation of the phosphoric acid radical was based on the work of Irvine and Scott[2] on the structure of diacetone glucose. At that time we had overlooked a later publication from Irvine's laboratory in which the previous theory of the structure of diacetone glucose was revised. The positions therein assigned by Macdonald[3] to the acetones are 1, 2- and 5, 6-, hence the benzoyl derivative obtained from it is 1,2- 5,6- diacetone-3-benzoyl glucose.

On this basis, the two phosphoric acid esters, one derived from the diacetone glucose, the other from the diacetone monobenzoyl derivative have the following structure: the former 1,2- 5,6-diacetone-3-phosphoric acid glucose and the latter 1, 2-acetone-3-benzoyl-5- or 6-phosphoric acid glucose.

It seems a strange coincidence that Karrer and Hurwitz[4] also overlooked the work of Macdonald and recently published a paper in which they dealt with the structure of diacetone glucose and assigned to it the same structure which had been formulated by Macdonald.

According to the present formulation the more resistant compound is the one in which the phosphoric acid radical is in position

[1] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1921, xlviii, 233.
[2] Irvine, J. C., and Scott, J. P., *J. Chem. Soc.*, 1913, ciii, 568.
[3] Macdonald, J. L. A., *J. Chem. Soc.*, 1913, ciii, 1896.
[4] Karrer, P., and Hurwitz, O., *Helvetica Chim. Acta*, 1921, iv, 728.

5 or 6, probably in position 6. This is in harmony with the pre-
vious observations on the rate of hydrolysis of 5-phosphoric acid
ribose. The latter was found very resistant to hydrolytic agents.

At the time when the allocation 1,2- 3,5 was assigned to the
acetones in the diacetone glucose the position 5,6 was assigned
to the benzaldehyde in benzylidene glucoside. Hence the re-
spective positions of the inorganic acid in the phosphoric esters
of the two sugar derivative should be different. These considera-
tions led us to prepare the substance.

On the basis of the latest formulation the second acetone in the
diacetone glucose and the benzaldehyde in the benzylidene mono-
acetone glucose should each enter into positions 5, 6. Hence the
phosphoric acid derivative of diacetone glucose and that of
1,2-acetone and 5,6-benzylidene glucose should both have the
phosphoric acid in position 3. To our surprise the constant of
hydrolysis of the phosphoric ester obtained from the benzylidene
derivative was found $17(10^{-3})$, which is of the same order of
magnitude as that of the phosphoric acid derivative obtained
from 1,2-acetone-3-benzoyl glucose, whereas a constant of about
$54(10^{-3})$ was expected.

A simple explanation for this unexpected result may be found
in the assumption that in the process of preparation the benzalde-
hyde is cleaved off before the phosphoric acid combines with the
glucose. This possibility requires special consideration in view
of the fact that the product obtained from benzylidene acetone
glucose was acetone phosphoric acid glucose. Thus the ben-
zaldehyde group was lost in the process of preparation. There
is reason to believe that the cleavage of the benzaldehyde took
place after the union with phosphoric acid was accomplished.
Had the cleavage of the benzaldehyde been the first step in the
operation, then the resulting substance would be 1,2-monoace-
tone glucose; however, when this compound is acted upon by
phosphorous oxychloride a product is obtained which has a con-
stant of hydrolysis $= 44(10^{-3})$. Furthermore, when diacetone
glucose is treated with phosphorous oxychloride two esters are
obtained, one of diacetone glucose and the second of monoacetone
glucose. The constants of hydrolysis of both are practically
identical, namely $56(10^{-3})$ and $58(10^{-3})$, thus showing that the
position of the phosphoric acid in both is identical and hence

indicating that the cleavage of the acetone took place after the phosphoric acid had united with the glucose.

A priori two other alternative explanations are possible. One is that the position of the benzaldehyde in the benzylidene acetone glucose is not on carbon atoms 5 and 6, the second that in the course of the reaction the phosphoric acid radical wanders from position 3 to either 5 or 6. Further investigations will deal with this problem.

The findings on the phosphoric esters of the methylated sugars are also now seen in a different light. The abnormal course of the hydrolysis of 2-phosphoric acid-3,5,6-trimethyl-methyl glucoside is apparently due to the fact that the substance contained a very small admixture of 6-phosphoric acid -2, 3, 5-trimethyl-methyl glucoside. The hydrolysis of the principal substance was undoubtedly completed in the first 60 minutes. Its constant of hydrolysis is not less than $84(10^{-3})$ or $87(10^{-3})$.

The following table contains the rates of hydrolysis of the phosphoric esters of substituted glucoses with special reference to the allocation of the phosphoric acid.

		K
1	5- or 6 (?)-phosphoric acid methyl glucoside (from α-methyl glucoside)...............................	$22(10^{-3})$
2	Mixture of 3-phosphoric acid with 5- or 6-phosphoric acid monoacetone glucose (from monoacetone glucose).....................................	$44(10^{-3})$
3	3-phosphoric acid-1,2- 5,6-diacetone glucose (from diacetone glucose)...............................	$56(10^{-3})$
4	3-phosphoric acid-1, 2-monoacetone glucose (by-product from diacetone glucose)....................	$58(10^{-3})$
5	5- or 6-phosphoric acid-3-monobenzoyl-1,2-acetone glucose (from 1,2-monoacetone-3-monobenzoyl glucose)..	$18(10^{-3})$
6	5- or 6-phosphoric acid-1,2-acetone glucose (from 5 by removing benzoyl group)......................	$24(10^{-3})$
7	5- or 6 (?)-phosphoric acid-1,2-acetone glucose (from benzylidene monoacetone glucose)................	$17(10^{-3})$
8	6-phosphoric acid-2, 3, 5-trimethyl-methyl glucoside.	$44(10^{-3})$
9	2-phosphoric acid-3, 5, 6-trimethyl-methyl glucoside.	$87(10^{-3})$

EXPERIMENTAL.

Benzylidene Monoacetone Glucose.

50 gm. of monoacetone glucose are heated with 300 cc. of freshly distilled benzaldehyde and 50 gm. of anhydrous sodium sulfate at 145°C. for 5 hours. While still warm, the solution is filtered into a distilling flask and the larger part of the benzaldehyde removed by distillation under diminished pressure. When the contents of the flask show a tendency to gelatinize, they are poured into 1 liter of ligroin (80–90°). On vigorous stirring the oily mass soon crystallizes. The crude product is filtered and washed with cold dry ether, in which it is practically insoluble. A nearly pure white product is obtained. Recrystallized several times from absolute alcohol this melts at 141-142°C. The yield is 20 gm.

0.1008 gm. substance: 0.2306 CO_2 and 0.0628 gm. H_2O.
$C_{16}H_{20}O_6$.   Calculated.   C 62.3, H 6.48.
          Found.   " 62.38, " 6.97.

The substance had the following rotation:

$$[\alpha]_D^{25} = \frac{+ 0.44° \times 100}{2 \times 1} = + 22°.$$

1, 2-Monoacetone-6-Phosphoric Acid Glucose from Benzylidene Monoacetone Glucose.

20 gm. of dried benzylidene monoacetone glucose are dissolved in 75 cc. of dry pyridine and cooled to −20°C. To this is added at once, a solution of 10 gm. of phosphorous oxychloride in 25 cc. of pyridine. The temperature of the reaction mixture does not go above +20°C. and crystals of pyridine hydrochloride settle out immediately on cooling. If the oxychloride solution is added too slowly the reaction is incomplete and only a small quantity of pyridine hydrochloride separates. After allowing the reaction mixture to stand for 1 hour, cold water is added. After the addition of 120 gm. of barium hydroxide, the pyridine is removed by distillation under diminished pressure. The residue is then neutralized with sulfuric acid until just acid to Congo red, shaken for ½ hour with 60 gm. of silver sulfate, and filtered. The filtrate is treated with hydrogen sulfide, the resulting precipitate removed by filtration. After removing the hydrogen sulfide by

a current of air the solution is again made alkaline with barium hydroxide. The excess barium is removed by means of carbon dioxide and the filtrate concentrated under diminished pressure to a syrup. This is taken up in absolute alcohol, filtered, and the barium salt precipitated in a large volume of dry ether. The substance analyzed for a monoacetone phosphoric acid glucose.

0.1104 gm. substance: 0.1156 gm. CO_2, 0.042 gm. H_2O, and 0.0481 gm. ash.
0.2916 "   "   : 0.0746 " $Mg_2P_2O_7$.
0.0972 "   "   : 0.0400 " $BaSO_4$.
$C_9H_{16}O_6H_2PO_3$ Calculated.   C 35.75, H 6.00, P 11.28.
           Found (calculated Ba-free). " 37.7, " 5.46, " 9.45.

Monoacetone Phosphoric Acid Glucose from Monoacetone Benzylidene Glucose.

7.271 gm. of the barium salt of this substance were dissolved in water and the volume was made up to 50 cc. Of this solution 3 cc., equivalent to 0.03 gm. of P, were put into glass tubes together with 2.1 cc. of N H_2SO_4 and 0.9 cc. of water and sealed. The tubes were heated at 100°C. for the intervals indicated in the following table. The contents of each tube were made up to 100 cc. and the P in 40 cc. portions was determined.

5-6 (?)-Phosphoric Acid-1, 2-Acetone Glucose.

Time.	$Mg_2P_2O_7$ in 40 cc.	Average.	P in $Mg_2P_2O_7$ in 100 cc.	P in free acid.	P of total P.	x	$a - x$	K
min.				per cent	per cent	$\frac{gm.}{Mg_2P_2O_7}$	$\frac{gm.}{Mg_2P_2O_7}$	$\frac{1}{t}\log\frac{a}{a-x}$
120	0.0018	0.0020	0.0014	0.43	4.6	0.0050	0.1027	0.000176
	0.0022							
240	0.0044	0.0030	0.0030	0.99	10.0	0.0107	0.0970	0.000182
	0.0042							
360	0.0060	0.0055	0.0038	1.19	12.8	0.0138	0.0939	0.000166
	0.0050							
480	0.0072		0.0050	1.57	16.8	0.0181	0.0896	0.000167

$a = 0.1077$

SULFURIC ESTERS OF SOME SUBSTITUTED GLUCOSES AND THEIR RATE OF HYDROLYSIS.

BY P. A. LEVENE AND G. M. MEYER.

WITH THE ASSISTANCE OF I. WEBER.

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It has been demonstrated in this laboratory that all known glucoproteins are protein derivatives of complex substances which are essentially sulfuric acid esters of disaccharides. It has been observed that individual esters of this group differ in their stability. In some of them the sulfuric acid is removed from the disaccharide very readily, whereas in other compounds it is removed with greater difficulty. *A priori* it seemed possible to explain the differences in stability of individual compounds by the difference in the position of the sulfuric acid on the glucose molecule. In this respect the behavior of the conjugated sulfuric acids resembles that of the conjugated phosphoric acids. In regard to the phosphoric esters of glucose,[1] it was demonstrated experimentally that their resistance is determined by the position of the phosphoric acid on the sugar molecule.

These considerations led us to synthesize two sulfuric acid sugar derivatives, one from diacetone glucose and the other from 1,2-acetone-3-benzoyl glucose. The first should yield a substance with the sulfuric acid attached to carbon atom 3, the second to either carbon atom 5 or 6.

For the preparation of sulfuric acid esters of glucose several methods have been recommended by Neuberg and Pollak,[2] and Neuberg and Liebermann.[3] They employed for the purpose either pyrosulfates or chlorosulfonic acid. Neuberg and Pollak have also

[1] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1922, liii, 431.
[2] Neuberg, C., and Pollak, H., *Biochem. Z.*, 1910, xxvi, 515.
[3] Neuberg, C., and Liebermann, L., *Biochem. Z.*, 1921, cxxi, 326.

mentioned that sulfuryl chloride may be used for this purpose. However, for the use of this reagent no experimental directions are given by them. Helferich[4] has also worked with sulfuryl chloride and obtained a dichlorohydrinsulfate. In the present experiments sulfuryl chloride was employed. The success of the synthesis depends largely on the temperature maintained during the operation. It was found advantageous to add the solution of sulfuryl chloride in chloroform to a pyridine solution of the sugar derivative cooled to $-10°$C., and to allow the temperature to rise to about 30°C. The details of the preparation are given in the experimental part.

Regarding the rates of hydrolysis it was found that the ester having the sulfuric acid in position 5 or 6 was more stable than the one having the acid in position 3 as may be seen from the following table.

$$K = \tfrac{1}{t}\log\frac{a}{a-x}$$

3-sulfuric acid-1, 2- 5, 6-diacetone glucose ....60 (10^{-3})

5- or 6-sulfuric acid-1, 2-acetone glucose.................40 (10^{-3})

This is in harmony with the views expressed in the publications from this laboratory on the structure of chondroitin and mucoitin sulfuric acids.

EXPERIMENTAL.

Sulfuric Acid Diacetone Glucose.

A solution of 10 gm. of diacetone glucose in 30 cc. of dry pyridine, is cooled to $-10°$C. To this is added a solution of 2.7 cc. of sulfuryl chloride in 25 cc. of dry chloroform, also cooled to $-10°$C. The temperature rises to 30°C., and the reaction product is allowed to stand at room temperature over night. The product is then dark red and contains no precipitate. Water and 60 gm. of barium hydroxide are then added and the chloroform and pyridine removed by distillation under diminished pressure. The solution is treated with sulfuric acid until it turns acid to Congo red and is shaken for $\tfrac{1}{2}$ hour with 30 gm. of silver sulfate. The excess silver is removed by hydrogen sulfide, which is then removed by a current of air. The excess of

[4] Helferich, B., *Ber. chem. Ges.*, 1921, liv, 1082.

barium is removed by means of carbon dioxide and the filtrate is concentrated to dryness under diminished pressure. The residue is dissolved in a small amount of absolute alcohol, the solution is filtered and precipitated in a large volume of dry ether.

For purification the precipitate is redissolved in absolute alcohol and reprecipitated in ether, this process is repeated several times.

10 gm. of diacetone glucose yield 5 gm. of the barium salt of sulfuric acid diacetone glucose.

The substance does not reduce Fehling's solution until after hydrolysis. Barium chloride, also, produces no precipitate in an aqueous solution of the substance. However, a precipitate of barium sulfate is formed after the substance is hydrolyzed with hydrochloric acid.

The elementary composition of the substance is not so good as is desired. However, taking into consideration the amorphous nature of the substances and their great solubility, the result may be regarded as satisfactory.

0.1096 gm. substance: 0.1334 gm. CO_2 and 0.0454 gm. H_2O.
0.2766 "   "   : 0.1320 " $BaSO_4$ (S determination).
0.0922 "   "   : 0.0254 "   "   (Ba   "   ).
$(C_{12}H_{20}O_9S)_2$ Ba. Calculated. C 35.5, H 4.66, S 7.88, Ba 16.85.
                     Found.   " 33.19, " 4.63, " 6.55, " 16.21.

5- or 6 (?)-Sulfuric Acid-Monoacetone Glucose from 3-Benzoyl-1, 2-Monoacetone Glucose.

Benzoyl monoacetone glucose (10 gm.) dissolved in 30 cc. of dry pyridine was reacted on with 2.7 cc. of sulfuryl chloride dissolved in 25 cc. of chloroform, as previously described under sulfuric acid diacetone.

The final residue was dissolved in a small quantity of hot absolute alcohol. On cooling barium benzoate crystallized. This was filtered off and the filtrate precipitated in a large volume of dry ether. This process was repeated several times.

The substance obtained analyzed for sulfuric acid monoacetone. It does not reduce Fehling's solution. An aqueous solution of the substance gives no precipitate with barium chloride until after hydrolysis with acid.

0.1062 gm. substance: 0.1136 gm. CO_2, 0.0352 gm. H_2O, and 40.3 gm. ash.
0.2912 "   "   : 0.1534 " $BaSO_4$ (S determination).
0.0971 "   "   : 0.0390 "   "   (Ba   "   ).
$(C_9H_{15}O_9S)_2$ Ba. Calculated. C 29.4, H 4.3, S 8.7, Ba 18.35.
                   Found.   " 29.17 " 3.7, " 7.23 "   23.63.

Sulfuric Acid Diacetone Glucose.

3.817 gm. of the barium salt of this substance were dissolved
in a little water and the volume was made up to 25 cc. Of this
solution 3 cc., equivalent to 0.031 gm. of S, were put into glass
tubes together with 1.68 cc. of N HCl and 4.32 cc. of H_2O. After
the tubes were sealed they were heated at 75°C. for the time
intervals indicated in the following table. The sulfuric acid was
determined as $BaSO_4$.

Time.	$BaSO_4$	S in $BaSO_4$.	S in free acid.	S of total S.	x	$a - x$	K
min.	gm.	gm.	per cent	per cent	gm. $BaSO_4$	gm. $BaSO_4$	$\frac{1}{t} \log \frac{a}{a-x}$
60	0.0153	0.0021	0.547	7.00	0.0153	0.2032	0.00053
240	0.0620	0.0085	2.22	28.4	0.0620	0.1565	0.00060
360	0.0857	0.0118	3.07	39.2	0.0857	0.1328	0.00060

$a = 0.2184$

Sulfuric Acid Monoacetone Glucose.

3.481 gm. of the barium salt of this substance were dissolved
in a little water and the volume was made up to 25 cc. Of this
solution 3 cc., equivalent to 0.031 gm. of S, were put into glass
tubes together with 1.94 cc. of N HCl and 4.06 cc. of H_2O. After
the tubes were sealed they were heated at 75°C. for the time inter-
vals indicated in the following table. The sulfuric acid was de-
termined as $BaSO_4$.

Time.	$BaSO_4$	S in $BaSO_4$.	S in free acid.	S of total S.	x	$a - x$	K
min.	gm.	gm.	per cent	per cent	gm. $BaSO_4$	gm. $BaSO_4$	$\frac{1}{t} \log \frac{a}{a-x}$
120	0.0119	0.0016	0.502	5.45	0.0119	0.2066	0.00040
240	0.0480	0.0066	2.02	22.0	0.0480	0.1705	0.00044
360	0.0622	0.0085	2.62	28.5	0.0622	0.1563	0.00040

PREPARATION AND ANALYSIS OF ANIMAL NUCLEIC ACID.

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The preparation of a pure animal nucleic acid remained a difficult task notwithstanding the fact that several methods have been recommended in recent years. A year ago the present writer made an effort to prepare animal nucleic acid by his picric acid method, slightly modifying the details of the procedure. The result was quite satisfactory when small quantities of the material were prepared by the writer personally. However, when the preparation of the material was left in the hands of technical assistants the product obtained by them was rather impure and besides had a brownish color which made the measurement of its optical activity very difficult. A new effort was made to work out an easy and reliable process. Preliminary to this attempt we tested all the recently published methods of W. Jones,[1] R. Feulgen,[2] and E. J. Baumann.[3] None proved satisfactory, since every sample prepared by any one of these methods gave a very marked biuret reaction. That the authors themselves obtained satisfactory results by their respective methods is not doubted, but we affirm that such results, to say the least, are not constant. Material prepared by the method of Jones[1] by a French firm also proved unsatisfactory.

After several trials we finally modified our older method by substituting colloidal iron for the picric acid.

The details of the process as carried out at present are as follows: The glands (10 lbs.) are dissected free from fat, ground in a chopping machine, and transferred into 5 liters of water,

[1] Jones, W., Nucleic acids; their chemical properties and physiological conduct, New York, 2nd edition, 1920.

[2] Feulgen, R., *Z. physiol. Chem.*, 1917–18, ci, 296.

[3] Baumann, E. J., *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 118.

containing 250 gm. of sodium hydroxide.   The mixture is boiled
for 35 minutes and then neutralized with acetic acid.   50 cc. of a
colloidal iron solution (iron dialyzed, 5 per cent Fe_2O_3, Merck)
are added and the solution is filtered and allowed to stand over
night.   To the filtrate is then added a double volume of methyl
alcohol containing 2 per cent of hydrochloric acid.   The pre-
cipitate thus formed is filtered off and washed with methyl alcohol
until the filtrate is free from hydrochloric acid.

This process has been applied to the preparation of nucleic
acid from thymus gland, spleen, kidney, pancreas, and liver.
The average respective yields from 10 lbs. of gland were: thymus,
150.0 gm.; spleen, 40.0 gm.; kidney, 25.0 gm.; pancreas, 35.0 gm.;
and liver, 18.0 gm.

From the first four organs the first precipitation yields a ma-
terial which is either entirely free, or contains barely detectable
traces, of biuret-giving substances.   In the last case one repre- ·
cipitation with alcohol containing 2 per cent hydrochloric acid
suffices to purify the material.   The material obtained from the
liver contains a marked proportion of glycogen.   Such prepara-
tions are readily purified by dissolving them in water with the
aid of a minimum amount of alkali, slightly acidulating with
hydrochloric acid, and precipitating the nucleic acid with a
20 per cent solution of cupric chloride.   The precipitate is washed
with water, suspended in alcohol containing 2 per cent hydro-
chloric acid, and carefully triturated until the larger part of the
copper is recovered.   The precipitate is then dissolved in water,
adding a minimum amount of alkali, and the nucleic acid is
precipitated by a double volume of alcohol containing 2 per cent
hydrochloric acid.   When this process fails, the crude nucleic acid
may be dissolved in water with the aid of a minimum amount of
alkali and precipitated with hydrochloric acid.   This precipitate is
then redissolved in water, by the addition of a minimum amount
of alkali and to the solution an equal volume of alcohol, containing
2 per cent of hydrochloric acid, is added.

The following table contains the results of the analysis of
various samples obtained by this process:

	C	H	N	P
Thymus gland...................	36.72	4.58	14.59	9.05
Spleen.........................	36.21	4.30	14.54	8.94
Kidney........................	36.51	4.17	14.07	8.74
Pancreas.......................	36.34	4.40	14.37	9.10
Liver..........................	35.69	4.05	15.25	9.05
Calculated for a hexose tetra-nucleotide......................	36.30	4.19	14.79	8.73

The agreement of the analytical results with the theory is quite satisfactory. However, not too much importance should be attached to it, since the elementary composition of amorphous substance has only a relative theoretical importance. Besides, not all samples gave identical analytical results. Often the original material contained 10 per cent of phosphorus, and about 12 per cent of nitrogen, but showed little deviation from the theory in the content of carbon and hydrogen. On the other hand, the table is very important in showing that nucleic acids derived from different organs do not vary in their elementary composition notwithstanding the claims to the contrary recently expressed by Feulgen and others.

Also as regards the content of purine bases, the acids from the various organs seem to show only such variations as could be expected from the degree of accuracy of the analytical methods. Thus the theory of the tetranucleotide requires for adenine picrate 27.10 per cent and for guanine 10.6 per cent purine bases.

The results of the analysis were as follows:

	Adenine picrate.	Guanine.
Thymus gland...........................	26.6	11.8
Spleen.................................	26.6	13.0
Kidney................................	23.3	12.6
Pancreas...............................	23.0	11.5
Liver.................................	30.0	11.8

ANALYTICAL PART.

The hydrolysis of the nucleic acids for the purpose of estimating the purine bases was carried out in the same way as described in a previous article, with the exception that instead of absolute

methyl alcohol, one containing 5 per cent of water was employed. The nucleic acid (50 gm.) is suspended in 500 cc. of 95 per cent methyl alcohol and hydrogen chloride gas is passed for 2 hours. The acid soon dissolves and gradually the hydrochlorides of the bases settle out.   To complete the separation the reaction product is allowed to stand over night.

Separation and Purification of the Purine Bases.—After a scrutiny of the analytical data on the nucleic acids published by different writers one is left with the impression that the workers encountered difficulties in obtaining the bases in analytically pure condition.   This is particularly true regarding guanine.   In part this difficulty arose from the method of hydrolysis, in part also from the method of isolating the bases.   It is possible, however, to prepare the bases in pure condition in a very short time. The process employed in the course of this work was the following:

1. Separation of Adenine from Guanine.—The hydrochlorides are dissolved in hot water and the solution is neutralized with sodium hydroxide until neutral to Congo red.   The guanine then precipitated out.   A precipitate is then formed which consists practically of pure guanine, slightly contaminated with adenine. In order to remove this it is again dissolved in dilute hydrochloric acid and again precipitated with sodium hydroxide.   The two filtrates are combined and the adenine is precipitated in the form of the picrate.   In the course of the present work the crude adenine picrate and the crude guanine were dried to constant weight.   The crude adenine picrate contains about 28 per cent of nitrogen whereas 29.3 per cent is required by theory and the guanine contained about 40 per cent of nitrogen whereas 46.35 per cent is required by theory.

2. Purification of the Base.—Adenine picrate is obtained analytically pure by one recrystallization from 25 per cent solution of acetic acid.   The crude picrate (10 to 12 gm.) is suspended in 1 liter of the acid, and boiled until dissolved.

Guanine is obtained analytically pure in the following manner. The crude material is dissolved in boiling dilute sulfuric acid and precipitated by means of silver sulfate.   The precipitate of guanine silver sulfate is filtered off while the reaction product is still hot. The silver salt is decomposed by means of hydrochloric acid and the clear filtrate from silver chloride is neutralized with 10 per

cent of sodium hydroxide. Free guanine is thus precipitated. On some occasions it was found necessary to repeat the silver precipitation process.

Analysis of Individual Nucleic Acids.

Thymus Gland.

0.1184 gm. substance: 0.1384 gm. CO_2 and 0.0420 gm. H_2O.
0.1736 "   "   required (Kjeldahl) 18.10 cc. 0.1 N acid.
0.2605 "   "   : 0.0846 gm. $Mg_2P_2O_7$.
               Found.   C 36.72, H 4.58, N 14.59, P 9.05.

Bases.—45.0 gm. of the dry material yield 12.0 gm. (26.6 per cent) of crude adenine picrate and 5.3 gm. (11.8 per cent) of crude guanine, containing 40 per cent nitrogen.

Adenine picrate analyzed as follows:

0.1000 gm. required (Kjeldahl, reduction with zinc) 20.75 cc. 0.1 N acid.
     $C_5H_5N_5C_6H_2(NO_2)_3$ OH + H_2O.   Calculated.   N 29.31.
                              Found.   " 29.05.

Guanine was analyzed as the free base.

0.0983 gm. substance required (Kjeldahl) 32.25 cc. 0.1 N acid.
               $C_5H_5N_5O$.   Calculated.   N 46.35.
                    Found.   " 45.93.

Spleen Nucleic Acid.

0.1006 gm. substance: 0.1444 gm. CO_2 and 0.0418 gm. H_2O.
0.1795 "   "   required (Kjeldahl) 18.65 cc. 0.1 N acid.
0.2693 "   "   : 0.0864 gm. $Mg_2P_2O_7$.
               Found.   C 36.21, H 4.30, N 14.54, P 8.94.

Bases.—45.0 gm. of the dry acid gave 12.0 gm. (26 per cent) of crude adenine picrate and 6.0 gm. (13.3 per cent) of crude guanine, having 39.2 per cent of nitrogen.

Adenine was purified by recrystallization and analyzed as follows:

0.1000 gm. substance: (Dumas) 26 cc. nitrogen at 26°C., 756 mm.
     $C_5H_5N_5$. $C_6H_2(NO_2)_3$ OH+H_2O.   Calculated.   N 29.31.
                              Found.   " 29.51.

Guanine was analyzed both as the free base and as the picrate.

0.0988 gm. substance required (Kjeldahl) 32.35 cc. 0.1 N acid.

C₅H₅N₅O. Calculated. N 46.35.
                Found.   " 45.84.

This substance was dissolved in water on addition of the required amount of 10 per cent sodium hydroxide. An excess of picric acid was then added. The picrate settled out gradually in the form of long needles and analyzed as follows:

0.1000 gm. substance: (Dumas) 24.2 cc. nitrogen at 23°C., 763 mm.

C₅H₅N₅O. C₆H₂(NO₂)₃ OH+H₂O. Calculated. N 28.13.
                                Found.   " 28.02.

Kidney Nucleic Acid.

0.0992 gm. substance: 0.1312 gm. CO₂ and 0.0378 gm. H₂O.
0.1693 "   "   required (Kjeldahl) 16.95 cc. 0.1 N acid.
0.2539 "   "   : 0.0834 gm. Mg₂P₂O₇.
              Found.   C 36.06, H 4.26, N 14.01, P 9.15.

Bases.—42.0 gm. of the substance yielded 9.8 gm. (23.3 per cent) of adenine picrate and 5.3 gm. (12.6 per cent) of crude guanine (N = 39.05 per cent).

Adenine picrate was purified by recrystallization and analyzed as follows:

0.1000 gm. substance: (Dumas) 25.3 cc. nitrogen gas at 23°C., 758 mm.

C₅H₅N₅. C₆H₂(NO₂)₃ OH+H₂O. Calculated. N 29.31.
                            Found.   " 29.10.

Guanine was analyzed as the sulfate.

0.0967 gm. of the dry substance required (Kjeldahl) 22.65 cc. 0.1 N acid.

(C₅H₅N₅O)₂ H₂SO₄. Calculated. N 34.99.
                    Found.   " 34.96.

Pancreas Nucleic Acid.

0.1037 gm. substance: 0.1382 gm. CO₂ and 0.0408 gm. H₂O.
0.1866 "   "   required (Kjeldahl) 19.15 cc. 0.1 N acid.
0.2799 "   "   : 0.0914 gm. Mg₂P₂O₇.
              Found.   C 36.34, H 4.40, N 14.37, P 9.10.

Bases.—The crude substance (35.0 gm.) gave 8.0 gm. (23 per cent) of crude adenine picrate and 4.0 gm. (11.5 per cent) of crude guanine.

Adenine picrate was recrystallized once and analyzed as follows:

0.1000 gm. substance: (Dumas) 26 cc. nitrogen at 26°C., 752 mm.
$C_5H_5N_5$. $C_6H_3(NO_2)_3$ $OH+H_2O$. Calculated. N 29.31.
Found. " 29.33.

Guanine was identified as the free base.

0.0979 gm. substance required (Kjeldahl) 32.10 cc. 0.1 N acid.
$C_5H_5N_5O$. Calculated. N 46.35.
Found. " 45.90.

Liver Nucleic Acid.

0.1094 gm. substance: 0.1432 gm. CO_2 and 0.0396 gm. H_2O.
0.1800 " "   required (Kjeldahl) 18.55 cc. 0.1 N acid.
0.2000 " "   : 0.0924 gm. $Mg_2P_2O_7$.
Found. C 35.69, H 4.05, N 15.25, P 9.05.

Bases.—The crude acid (33 gm.) gave 9.0 gm. (30 per cent) of adenine picrate and 3.9 gm. (11.8 per cent) of crude guanine (by error was not analyzed).

Adenine picrate was recrystallized once and analyzed as follows:

0.1000 gm. substance: (Dumas) 26.4 cc. nitrogen at 29°C., 753 mm.
$C_5H_5N_5$. $C_6H_3(NO_2)_3$ $OH+H_2O$. Calculated. N 29.31.
Found. " 29.57.

Guanine was analyzed as the free base.

0.0963 gm. substance required (Kjeldahl) 31.75 cc. 0.1 N acid.
$C_5H_5N_5O$. Calculated. N 46.35.
Found. " 46.15.

BENZYLIDENE-ETHYL-CHITOSAMINATE AND BENZYLIDENE-ETHYL-DIAZOGLUCONATE (MANNONATE).

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Levene and La Forge[1] observed that benzylidene-ethyl-chitosaminate hydrochloride on cautious treatment with sodium nitrite, is converted into the corresponding diazo derivative. On the other hand, it has been known that chitosaminic acid and chitosamine are converted by the same reagent, not into a mixture of two epimeric deaminized derivatives, but each into a single derivative. To Levene and La Forge it seemed possible to regard the diazo derivative as an intermediate substance in the process of deamination. However, there existed no experimental proof showing the transformation of the diazo esters of the sugar acids into a single sugar acid and not into a pair of epimers.

There also existed no experimental data regarding the character of the substances which would result from the substitution of the diazo group by hydrogen chloride or bromide.

In the present work the diazo compound was hydrolyzed and the resulting hydroxy-acid identified, on the other hand, the diazo compound was converted into the bromo and chloro compounds and the chloro derivative again converted into a 2-aminohexonic acid.

On hydrolysis of the diazo derivative with dilute acetic acid the formation of only one derivative was observed; namely, benzylidene-ethyl-gluconate. This was identified in the form of saccharic acid. However, in one experiment, anhydrosaccharic acid was isolated. The bromo and chloro derivatives were prepared each with a constant melting point and constant specific

[1] Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1915, xxi, 345.

rotation, showing that in each instance only one substance and not a pair of epimers had been formed. The latter conclusion is further substantiated by the fact that the chloro derivative on treatment with ammonia gave but one chitosaminic acid (2-amino-mannonic) and not a mixture of two-aminohexonic acids. One may be inclined to explain these reactions by the presence in the molecule of three asymmetric carbon atoms, on the other hand, this asumption is not bindng since Fischer obtained two epimeric halogen derivatives, when bromine or chlorine was added to glucal.

Regarding the direction of the rotation of carbon atom 2 in the derivatives of the diazo compound, the following was observed. On hydrolysis of the compound with dilute acids a substance resulted in which the rotation of the carbon atom 2 was in the opposite direction from that of the carbon atom 2 of chito-saminic acid. On the other hand, in the bromo, chloro, and amino derivatives, the rotation of the carbon atom 2 was the same as in the original chitosaminic acid. It is here accepted that in the chloro and bromo derivative the direction of the rotation of the carbon atom 2 determines the direction of rotation of the acid. Hence for the present for the chloro and bromo derivatives, the configuration of mannonic acid is assumed. On this assumption chitosaminic acid passes through the diazo derivative into the chloro derivative and back into the amino-acid apparently without Walden inversion. On the other hand, deamination through only the diazo derivative undergoes the Walden inversion in the same way as on direct deamination.

In connection with the Walden inversion it is interesting to note that it occurs in this instance both in the acid and in its ester. In the majority of amino-acids according to the observation of Fischer, the inversion occurs only in the acid and not in the ester. In the amino-acids where acid and ester both gave rise to the same hydroxy-acid, Fischer's original view was that no inversion occurred in either, and later he reversed his opinion accepting an inversion in both. In the present instance chito-saminic acid and ester give a hydroxy-acid in which the carbon atom 2 rotates to the right, whereas chitosamine leads to a hy-droxy-acid in which the carbon atom 2 rotates to the left. On the basis of considerations discussed in another article it was as-

sumed that the inversion occurred in chitosaminic acid, hence it also occurs in the ester. This fact is a corroboration of the later theory of Fischer.

The set of reactions here described are represented by the following graphic expression:

III.

IV.   V.

I.   II.
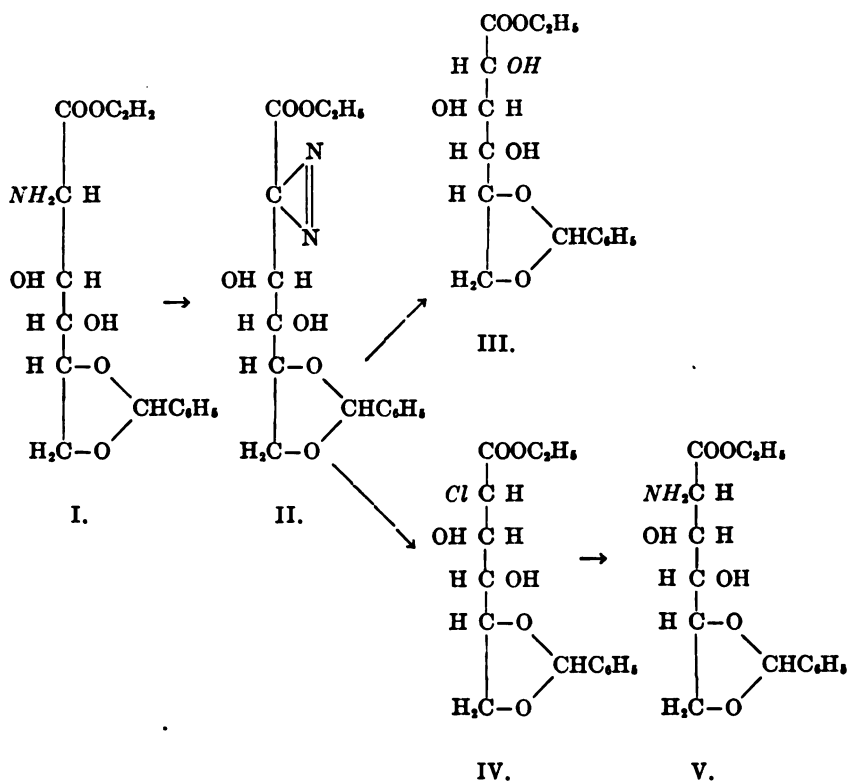
In the course of the work some derivatives were prepared which have no direct bearing on the present problem, but eventually they may become of theoretical value. These compounds are: Benzylidene-chitosaminic acid, benzylidene-ethyl-chitosaminate (this was reconverted into its hydrochloride), benzylidene-acetone-ethyl-chitosaminate, benzylidene-1-ethyl-2, 3-anhydrogluconate (mannonate), and benzylidene-ethyl-desoxygluconate (mannonate). The last may be a mixture of gluconate and mannonate.

Benzylidene-1-amino-2,3-dehydrogluconate   (mannonate)   and
benzylidene-1-amino-2-chloromannonate were also prepared.

The first three substances were obtained in the process of prep-
aration of free benzylidene-ethyl-chitosaminate. In one phase
of the work it seemed as if the halogen derivative of the diazo
compound was readily converted into the free amino derivative,
and hence it was desirable to compare the substance obtained
from the diazo compound with that from benzylidene-ethyl-
chitosaminate hydrochloride.

The benzylidene-acetone-ethyl-chitosaminate was obtained
accidentally, when it was attempted to recrystallize benzylidene-
ethyl-chitosaminate from acetone. The condensation took place
in the short time required to bring the original substance in
solution. It is remarkable that the acetone is cleaved off as
readily as it is condensed with the benzylidene-ethyl-chitosami-
nate. Benzylidene-chitosaminic acid was obtained as a by-
product in the process of preparation of its ester.

Benzylidene-1-ethyl-2,3-anhydrogluconate (mannonate) is ob-
tained almost instantly when an alcoholic solution of benzyli-
dene-ethyl-chitosaminate is poured into aqueous ammonia and
the solution cooled to 0°C. This derivative apparently retains
the enolic structure

$$CH_2 \cdot CH \cdot CHOH \cdot CHOH \cdot CH = CHO \cdot COOC_2H_5$$
$$\underset{\underset{CHC_6H_5}{O \diagup O}}{| \qquad |}$$

and not the structure

$$CH_2 \cdot CH \cdot CHOH \cdot CHOH \cdot CH_2 \cdot CO \cdot COOC_2H_5 \quad .$$
$$\underset{\underset{CHC_6H_5}{O \diagup O}}{| \qquad |}$$
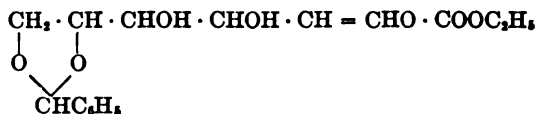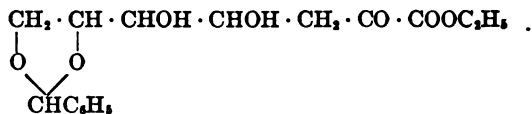
since with phenylhydrazine it did not form a hydrazone, and fur-
thermore in the presence of palladium it was readily hydrogenated
giving the following substance:

$$CH_2 \cdot CH \cdot CH(OH) \cdot CH(OH) \cdot CH_2 \cdot CHOH \cdot COOC_2H_5$$
$$\underset{\underset{CHC_6H_5}{O \diagup O}}{| \qquad |}$$
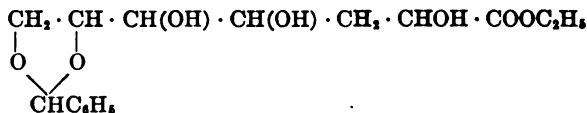
(The position of the desoxy carbon atom may be either 2 or 3.)

It is not known whether this is a single substance or a mixture of two epimers. Thus attempts to convert the bromo derivatives into benzylidene-chitosaminic ester failed. The attempt to convert the free bromomannonic acid into chitosaminic acid was also not successful.

Benzylidene-1-amino-2-chloromannonate is formed when an alcoholic solution of the chloro ester is poured into an excess of aqueous ammonia and the solution is allowed to crystallize. The same substance is formed when the chloro ester is dissolved in alcohol containing ammonia gas. On the contrary, if an alcoholic solution of the chloro ester added to aqueous ammonia is heated at 95°C. for 10 hours in a sealed tube the product is benzylidene-ethyl-chitosaminate.

Since in the course of the reaction some decomposition takes place it is more conveniently identified as chitosaminic acid.

EXPERIMENTAL.

Benzylidene-Ethyl-Chitosaminate Hydrochloride.—The substance was prepared under conditions previously described. For purification it was dissolved in methyl alcohol. To the solution dry ether was added until crystallization was complete. The substance melted at 200°C. (uncorrected) and analyzed as follows:

0.1972 gm. substance: (Kjeldahl) 5.65 cc. 0.1 N acid.
0.1972 " " : (Volhard) 5.6 " 0.1 N silver nitrate.
0.020 " " : (a) (Van Slyke) in 4 min. 0.38 cc. nitrogen at 22°C., 753.4 mm.
0.020 gm. substance: (b) (Van Slyke) in 30 min. 0.59 cc. nitrogen at 22°C., 753.4 mm.

$C_{15}H_2NO_7HCl.$ Calculated. N 4.04, Amino N 4.04, Cl 10.94.
　　　　　　　　　Found. " 4.01, " " (a) 1.06, " 10.07.
　　　　　　　　　　　　　　　　　　" " (b) 1.65.

The substance had the following rotation:

$$[\alpha]_D^{20} = \frac{-0.30° \times 100}{1 \times 1} = -30°$$

Benzylidene-Ethyl-Chitosaminate and Benzylidene-Chitosaminic Acid.—The hydrochloride (40.0 gm.) was dissolved in water (150 cc.) and an excess of an aqueous 1.0 N sodium hydroxide

solution (140 cc.) was added. On scratching the walls of the container the solution nearly solidifies into a mass consisting of curved needles. The precipitate was filtered on a suction funnel and washed a few times with water. The precipitate was dried under diminished pressure over sulfuric acid until practically dry. The yield of the dry product was about 20.0 gm. The substance consisted principally of the free benzylidene-chitosaminic ester with a small admixture of benzylidene-chitosaminic acid. The filtrate contained a larger quantity of the latter substance. This crystallized out on standing in the form of large crystalline plates. The separation of the benzylidene ester from the acid was accomplished in the following way: The dried substance (1 part) was taken up in boiling 98.5 per cent alcohol (4 parts) and filtered. The insoluble part consisted of the benzylidene-chitosaminic acid. The filtrate contained the ester. On standing it crystallized out in long prisms. For analysis it was recrystallized twice from small volumes of 98.5 per cent alcohol. It melted at 120°C. (corrected) and analyzed as follows:

0.0770 gm. substance: 0.1638 gm. CO_2 and 0.0432 gm. H_2O.
0.1288 " " required (Kjeldahl) 4.45 cc. 0.1 N acid.
$C_{15}H_{20}NO_6$. Calculated. C 57.85, H 6.80, N 4.50.
Found " 57.94, " 6.28, " 4.83.

The substance in methyl alcoholic solution had the following rotation:

$$[\alpha]_D^{20} = \frac{-0.50° \times 100}{1 \times 1} = -50°$$

The benzylidene-chitosaminic acid was purified by dissolving in hot water and adding 98.5 per cent alcohol to slight opalescence. On standing the substance crystallized in large prismatic plates. After two or three crystallizations the substance analyzed correctly. It melted at 230°C. (uncorrected) and analyzed as follows:

0.1084 gm. substance: 0.2008 gm. CO_2 and 0.0594 gm. H_2O.
0.1859 " " required (Kjeldahl) 6.55 cc. 0.1 N acid.
0.0186 " " : (Van Slyke) 1.75 cc. nitrogen at 24.0°C., 765 mm.
$C_{13}H_{17}NO_6$. Calculated. C 55.09, H 6.05, N 4.94, NH_2 4.94.
Found " 55.40, " 6.19, " 4.93, " 4.92.

The substance had in aqueous solution the following optical rotation:

$$[\alpha]_D^{20} = \frac{-0.28° \times 100}{1 \times 1} = 28°$$

Benzylidene-Acetone-Ethyl-Chitosaminate.—This substance was obtained, incidentally, in an experiment aiming to separate benzylidene-chitosaminic acid from its ester. The crude material (40.0 gm.) was suspended in dry acetone and digested on a boiling water bath. A part (3.0 gm.) remained undissolved. This substance proved to be the benzylidene-chitosaminic acid. The mother liquor was allowed to stand in a vacuum desiccator over sulfuric acid. Soon very large prismatic crystals began to form on the edge of the liquid and after a few days crystallization seemed complete. The yield was about 20 gm. From the mother liquor on standing, further crystallization took place. For purification the material may be recrystallized either from absolute alcohol or from acetone. The substance melts sharply at 128°C. It analyzed as follows:

0.1134 gm. substance: 0.2380 gm. CO_2 and 0.732 gm. H_2O.
0.1986 " " required (Kjeldahl) 5.55 cc. 0.1 N acid.
0.020 " " : (Van Slyke) after 30 min., 0.87 cc. nitrogen at 24°C., 760 mm.

$C_{18}H_{25}NO_6$. Calculated. C 61.50, H 7.18, N 3.98.
Found. " 61.37, " 7.22, " 3.91.

The optical rotation of the substance was:

$$[\alpha]_D^{20} = \frac{-0.70° \times 100}{1 \times 1} = -70°$$

The substance is readily reconverted into the hydrochloride of benzylidene-ethyl-chitosaminate. It (2.0 gm.) was dissolved in 20 cc. of dry methyl alcohol, to this solution 20 cc. of 0.6 N hydrochloric acid in dry ether were added, then dry ether until slight opalescence. On standing a substance crystallized which melted at 202°C. (uncorrected) and analyzed as follows:

0.1072 gm. substance: 0.2044 gm. CO_2 and 0.0626 gm. H_2O.
0.1932 " " required (Kjeldahl) 5.55 cc. 0.1 N acid.
. $C_{15}H_{22}NO_6HCl$. Calculated. C 51.72, H 6.34, N 4.04.
Found. " 51.75, " 6.50, " 3.99.

Diazo Derivative of Benzylidene-Ethyl-Chitonate.—The original method for the preparation of the diazo ester was slightly modified. The hydrochloride of benzylidene-ethyl-chitosaminate (20 gm.) is dissolved in 250 cc. of water and chilled to the same temperature. The two solutions are combined and to

the resulting solution glacial acetic acid (30.0 cc.) is added. The contents of the flask are practically solidified. The product at this phase seems colorless. When filtered off, however, it was a light yellow. The product is dissolved in ether and the ethereal solution is washed (four times) in a separatory funnel with a cold solution of sodium carbonate and then (three times) with water. The ethereal solution is dried by means of anhydrous sodium sulfate and concentrated under diminished pressure to a volume of about 35 cc., then transferred into an evaporating dish which is placed in a vacuum desiccator over sulfuric acid. The solution is evaporated to dryness leaving a light yellow mass which is readily pulverized. The powder is exhaustively extracted with low boiling petroleum ether and is then analytically pure.

The nitrogen estimation is easily carried out by the process described by Levene and Mikeska.[2]

Several samples were analyzed in this manner with the following results:

Sample 1. 0.020 gm. substance: 1.60 cc. nitrogen at 26°C., 760 mm.
"   2. 0.020 "   "   : 1.57 "   "   " 26° " 760 "
"   3. 0.020 "   "   : 1.54 "   "   " 26° " 760 "

$C_{16}H_{18}O_6N_2$. Calculated.   N 8.69.
                        Found, Sample 1.   " 8.88.
                                "   "   2.   " 8.71.
                                "   "   3.   " 8.55.

The optical rotation of the substance was the following:

$$[\alpha]_D^{20} = \frac{-1.00° \times 100}{2 \times 1} = -50°$$

Hydrolysis of the Diazo Derivative.—The product resulting from hydrolysis of the diazo derivative depends on the conditions of the reaction. If the reaction takes place in the absence of water or in organic solvents containing only a small proportion of water the product is apparently ethyl-2,3-anhydrogluconate, otherwise ethyl-gluconate or ethyl-1,4-anhydrogluconate is formed as the principal product. It was found difficult to isolate and identify the original reaction product, hence this was further oxidized by means of nitric acid. In the conditions in which only the unsaturated acid formed the product of oxidation was mesotartaric acid, under other conditions the product of oxidation was either

[2] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1922, lii, 485.

saccharic or anhydrosaccharic acid. The conditions of reaction were as follows:

The diazo compound (20 gm.) was suspended in 400 cc. of distilled water and glacial acetic acid (20.0 gm.) was added. The flask is placed near a hot water bath and from time to time warmed on the water bath so as to maintain a continuous but gentle evolution of nitrogen gas. The reaction was considered completed when a dark yellow oil (benzaldehyde) settled out on the bottom of the flask. The oil is separated in a separatory funnel, the aqueous portion is extracted with ether and concentrated to nearly dryness under diminished pressure. The residue is dissolved in water and again concentrated under diminished pressure. The operation is repeated four times. In this manner the greatest part of the acetic acid is removed. The final residue is taken up in 40.0 cc. of water, an equal volume of concentrated nitric acid is added, and the solution is allowed to stand over night. It is then transferred to a clock-glass and evaporated to dryness on a water bath. The residue is dissolved in nitric acid, diluted with an equal volume of water, and the solution evaporated to dryness. The residue is then dissolved in water and the process repeated. The final product is dissolved in water, again shaken out with ether, and finally converted into the calcium salt.

The yield of the calcium salt is about 5 to 6 gm. from 100 gm. of the diazo compound. In the earlier experiments the calcium salt was purified by repeatedly dissolving it in water containing the requisite amount of oxalic acid and reconverting it into the calcium salt until a product with a maximum optical rotation of about $[\alpha]_D^{20} = + 24.0°$ was obtained. Under these conditions anhydrosaccharic acid was obtained.

When the calcium salt was only once recrystallized and then converted into the acid potassium salt, the salt of saccharic acid was obtained.

The analytical results obtained on anhydrosaccharic acid were as follows:

0.1000 gm. substance: 0.0348 gm. K_2SO_4.

$$C_6H_7O_7Ka + H_2O. \quad \text{Calculated.} \quad K \ 15.70.$$
$$\text{Found.} \quad \text{`` } 15.61.$$

The optical rotation of the substance was:

$$[\alpha]_D^{20} = \frac{+ 0.63° \times 100}{1 \times 1} = + 63°$$

The analytical results obtained on the salt of the saccharic acid were as follows:

0.1000 gm. substance: 0.0352 gm. K_2SO_4.

C₆H₉O₈K.   Calculated. K 15.70.
$C_6H_9O_8K$.   Calculated. K 15.70.
                 Found.   " 15.80.

The optical rotation of the substance is as follows:

$$[\alpha]_D^{20} = \frac{+0.07° \times 100}{1 \times 1} = +7°$$

Benzylidene-1-Ethyl-2-Bromomannonate.—The diazo compound (20.0 gm.) is suspended in 400.0 cc. of anhydrous (over sodium) ether and dry ether saturated with hydrogen bromide gas is added as long as the diazo compound is dissolved and the yellow color has disappeared. The ethereal solution is then washed with a saturated solution of sodium carbonate, cooled to 0°C. The operation is repeated until all hydrobromic acid is removed from the ethereal solution. The ethereal solution is then dried with anhydrous sodium sulfate, concentrated to a small volume, and the bromo compound is precipitated with ligroin (80–90°C.). The crude compound is obtained analytically pure after several recrystallizations, from a mixture of ether and ligroin. The yield was between 25 to 40 gm. from 100.0 gm. of the diazo compound. The substance melted at 119°C. (corrected) and analyzed as follows:

0.1040 gm. substance: 0.1820 gm. CO_2 and 0.0480 gm. H_2O.
0.2000 "   "   : 0.1016 gm. AgBr.

C₁₆H₁₉BrO₆.   Calculated. C 47.99, H 5.10, B 21.30.
$C_{16}H_{19}BrO_6$.   Calculated. C 47.99, H 5.10, B 21.30.
                  Found.   " 47.25, " 5.16, " 21.56.

The optical rotation of the substance was:

$$[\alpha]_D^{20} = \frac{-0.66° \times 100}{1 \times 2} = -33°$$

Benzylidene-1-Ethyl-2,3-Anhydromannonate.—The substance was obtained in an experiment which aimed to convert the bromo compound into the corresponding amino derivative.

The bromo compound (30.0 gm.) is dissolved in 35 cc. of 98.5 per cent alcohol and poured into 30.0 cc. of concentrated aqueous ammonia. After standing for 5 to 10 minutes at room temperature the substance solidifies on cooling into a solid mass consisting of long microscopic needles. The material is filtered, dried in a vacuum desiccator over sulfuric acid and then recrystallized

from 35 per cent alcohol. This operation is repeated several times. Finally the product is twice recrystallized in its own weight of boiling 98.5 per cent alcohol. The final product melted at 122.5°C. (corrected) and analyzed as follows:

0.1130 gm. substance: 0.2522 gm. CO_2 and 0.0594 gm. H_2O.
$C_{16}H_{18}O_6$. Calculated. C 61.21, H 6.12.
Found. " 60.86, " 5.88.

The optical rotation of the substance was:

$$[\alpha]_D^{20} = \frac{-1.10° \times 100}{1 \times 1.5} = -73.3°$$

Benzylidene-Ethyl-Desoxygluconate (Mannonate).—The previous substance (93.0 gm.) was dissolved in 10 cc. of 98.5 per cent alcohol and saturated with hydrogen gas in the presence of Paal's colloidal palladium. The substance absorbed the theoretical volume of hydrogen. The operation was completed in 72 hours. The product was filtered. The greatest part of the palladium, however, remained in colloidal solution. Hence the filtrate was concentrated to dryness under diminished pressure. The residue was dissolved in a little boiling alcohol with charcoal, filtered, and allowed to evaporate to dryness. This operation was repeated three times when a perfectly colorless product was obtained. It melted at 126°C. (corrected) and analyzed as follows:

0.1081 gm. substance: 0.2274 gm. CO_2 and 0.0642 gm. H_2O.
$C_{16}H_{20}O_6$. Calculated. C 60.81, H 6.76.
Found. " 60.91, " 7.05.

The optical rotation of the substance was:

$$[\alpha]_D^{20} = \frac{-0.52° \times 100}{1 \times 2} = -26°$$

Benzylidene-1-Amino-2,3-Anhydrogluconate (Mannonate).—When a solution of the bromo compound in alcohol is added to ammonia water following exactly the same conditions as for the preparation of the benzylidene-ethyl-anhydrogluconate, and is allowed to stand at 0°C., the solution remains either liquid or partly gelatinous. On further cooling in an ice-alcohol mixture the contents of the flask turn into a gelatinous mass. This is best filtered and washed with water, it then acquires a white granular character. After it is dried in a vacuum desiccator (over soda-lime) to complete dryness it can be recrystallized from absolute alcohol. The

process may be repeated until the product is analytically pure. The substance melted at 230°C. and analyzed as follows:

0.1106 gm. substance: 0.2380 gm. CO_2 and 0.0586 gm. H_2O.
0.0993 "   "   required (Kjeldahl) 3.64 cc. 0.1 N acid.
$C_{13}H_{15}N O_5$. Calculated. C 58.84, H 5.70, N 5.28.
                Found.   " 58.68, " 5.93, " 5.14.

The substance had the following rotation:

$$[\alpha]_D^{20} = \frac{+1.30° \times 100}{1 \times 2} = +65°$$

Action of Aqueous Ammonia on Benzylidene-2-Bromo-Ethyl-Gluconate.—Benzylidene-2-bromo-ethyl-gluconate (5.0 gm.) was suspended in 100 cc. of 2 per cent sulfuric acid and placed on a water bath for 1 hour. The benzaldehyde settled out on the bottom of the flask in the form of an oil. The oil was extracted by means of ether and the aqueous solution freed from sulfuric acid quantitatively by means of barium hydroxide. The aqueous solution was then concentrated to a volume of 20 cc., an equal volume of concentrated ammonia water was added, and the solution was kept in a sealed tube at 100°C. for 24 hours. The resulting solution after the removal of the ammonia, showed the presence only of a few mm. of amino nitrogen.

Benzylidene-1-Ethyl-2-Chlorogluconate.—The substance was prepared in the same manner as the corresponding bromo compound, with the exception that ether saturated with hydrogen chloride gas was used. The yield was 10.0 gm. from 100.0 gm. of the diazo compound. It melted at 127°C. and analyzed as follows:

0.1062 gm. substance: 0.2122 gm. CO_2 and 0.0584 gm. H_2O.
0.2024 "   "   : 0.0858 " AgCl.
$C_{15}H_{19}Cl O_6$. Calculated. C 54.46, H 5.8, Cl 10.74.
                Found.   " 54.69, " 6.11, " 10.48.

The rotation of the substance was:

$$[\alpha]_D^{20} = \frac{-0.20° \times 100}{1 \times 1} = -20°$$

Benzylidene-1-Amino-2-Chloromannonate.—The substance described in the previous section (5.0 gm.) was dissolved in 5 cc. of 98.5 per cent alcohol and transferred into 4.0 cc. of aqueous ammonia. The solution was allowed to stand over night and then concentrated in a vacuum desiccator. A deposit, consisting of microscopic needles, was formed. This was recrystallized

first from 35 per cent alcohol and finally from 98.5 per cent alcohol until the substance gave a negative test with Nessler's reagent. The substances melted at 197°C. (corrected) and analyzed as follows:

0.1022 gm. substance: 0.1938 gm. CO_2 and 0.0540 gm. H_2O.
0.1857 " " : 0.0844 " AgCl.
0.1887 " " required (Kjeldahl) 5.95 cc. 0.1 N acid.
    $C_{15}H_{18}NO_6Cl$. Calculated. C 51.73, H 5.35, N 4.69, Cl 11.75.
               Found. " 51.72, " 5.52, " 4.41, " 11.23. .

The substance had the following rotation:

$$[\alpha]_D^{20} = \frac{-0.23° \times 100}{1 \times 1} = -23°$$

Conversion of the Benzylidene-1-Ethyl-2-Chloromannonate into Chitosaminic Acid.—The chloro compound (3.0 gm.) was dissolved in 4 cc. of 98.5 per cent alcohol and the solution added to 4.0 cc. of aqueous ammonia. This is sealed in a tube and heated at 95°C. for 10 hours. At the end of that time the tube is allowed to cool and the solution which has then turned dark brown is concentrated to nearly dryness under diminished pressure. The residue is taken up in 2 per cent sulfuric acid and boiled over a flame for 15 minutes. It is then allowed to cool and washed with ether, (in a separatory funnel) to remove the benzaldehyde. The resulting aqueous solution is freed from hydrochloric acid and ammonia in the usual way and concentrated to nearly dryness. The residue is dissolved in a little water, acetone is added to the solution until an oil settles out, and all is warmed on a water bath until crystallization begins. Prior to treatment with acetone a small sample of the aqueous solution was used for an amino nitrogen determination according to Van Slyke. On the basis of this estimation the solution contained 0.9 gm. of chitosaminic acid. However, from five experiments only 2.5 gm. of chitosaminic acid crystallized. After one recrystallization the substance was analytically pure. It analyzed as follows:

0.1108 gm. substance: 0.1508 gm. CO_2 and 0.0674 gm. H_2O.
0.0670 " " required (Kjeldahl) 3.45 cc. 0.1 N acid.
    $C_6H_{13}NO_6$. Calculated. C 36.92, H 6.66, N 7.18.
               Found. " 37.11, " 6.80, " 7.21.

The rotation of the substance was:

$$[\alpha]_D^{20} = \frac{-0.15° \times 100}{1 \times 1} = -15°$$

THE FATE OF SOME OF THE PHENYLACETYLATED AMINO-ACIDS IN THE ANIMAL ORGANISM.

By GEORGE J. SHIPLE AND CARL P. SHERWIN.

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Magnus-Levy endeavored (1) to account for the formation of the large quantities of glycocoll which the body can furnish after the ingestion of benzoic acid on the theory that hippuric acid might possibly originate by the curtailment, as it were, of longer chained benzoylated amino-acids. Accordingly, he benzoylated ten of the known amino-acids and injected the compounds subcutaneously into dogs. Quantitative analysis of the urine showed that in no case was there any evident increase in the amount of hippuric acid excreted. On the contrary, the investigator was able to recover the original substances from the urine, and this in such quantities that the absence of any such partial demolition of the longer chained amino-acids was assured. Ando (2) took α-aminocinnamic acid, which is known to be completely oxidized in the body, and benzoylated it. He found that both after ingestion and injection, it was eliminated in the urine unchanged. When, however, benzoylated tyrosine or benzoylated p-hydroxy-α-aminocinnamic acid was fed, these compounds were almost entirely destroyed in the organism.

It would seem therefore, that an aliphatic α-amino-acid, regardless of the length of its chain, is not subject to oxidation as long as the α-amino group remains intact, and that the same applies also to the aromatic amino-acids, unless as in the cases of tyrosine and p-hydroxy-α-aminocinnamic acid, the para position of the ring has been subjected to previous partial oxidation. Moreover, the evidence seems conclusive that the molecule in the above cases was rendered impervious to the attempts at oxidative or hydrolytic deamination by the replacement of one of the hydrogen atoms of the α-amino group by a benzoyl radical. Further-

more, as other investigators have shown, the same successful "blocking" of the α-amino group has been effected by the formation of the uramino compound or of the hydantoin. Thus Salkowski (3) showed that when hydantoic acid is fed to rabbits it is excreted as such in the urine; and Rohde (4) isolated the greater part of the uramino derivative of leucine from the urine of a cat after the intravenous injection of the substance. Finally, Lewis and Root (5) have recently shown that the phenyl-uramino derivative of cystine probably passes through the body without undergoing oxidation, for there is little increase in the amount of oxidized sulfur in the urine after the substance has been fed, and no increase in this form of sulfur after the injection of the substance. For further corroboration of this work the reader is referred to the very recent article of Hijikata (6) who has described in detail the "rediscovery" of some of the facts mentioned above.

In our work we had in mind four different problems: (a) the preparation and study of a number of the phenylacetyl derivatives of the natural amino-acids; (b) the determination of the efficiency of this phenylacetyl radical as a "block" to the catabolism of these amino-acids; (c) an attempt to solve the mystery regarding the genesis of glycocoll in the animal organism; and (d) a study of the physiological effects produced on one animal by the detoxication products elaborated by an animal of another species.

Accordingly, we prepared the phenylacetyl derivatives of glycocoll, alanine, leucine, glutamine, glutamic acid, asparagine, aspartic acid, and ornithine, according to the Schotten and Baumann method, namely by shaking a weakly alkaline solution of the amino-acid with phenylacetyl chloride. Of these compounds, phenylacetyl alanine and phenylacetyl leucine had never been prepared before. They were studied, therefore, in somewhat greater detail.

The phenylacetyl radical, differing chemically but little from its homologue, the benzoyl radical, was expected to act as a complete "block" of the amino group, and therefore to furnish little additional evidence or information in this respect above that supplied by the results obtained by Magnus-Levy. In one way, however, it seemed to offer a new and interesting clue to the solution of

the mystery. Benzoic acid combines with glycocoll in the human organism as well as in that of the lower animals and is excreted in the urine of both as hippuric acid. Phenylacetic acid, however, combines in the human body with the longer chained amino-acid glutamine, and is excreted as phenylacetyl glutamine or phenyl-acetyl glutamine urea, but in the bodies of the lower animals it simply combines with glycocoll and appears in the urine as phenaceturic acid. It seemed that this phenylacetyl glutamine in the brute organism might possibly be an intermediary product in the detoxication of phenylacetic acid, which product is then subjected to further decomposition before elimination, with the formation of phenaceturic acid. In other words, the splitting off of three carbon atoms from the glutamine molecule would result in the production of glycocoll. This idea was further strengthened by the fact which was found later that glutamine as well as glyco-coll can be made synthetically by the human body at the expense of nitrogen which otherwise would have appeared in the urea fraction (7). Thus a man placed on a carbohydrate diet and reduced to a condition of endogenous protein catabolism was fed phenylacetic acid. It was found that his urea nitrogen dropped from about 75 to 28 per cent of the total nitrogen. After feeding benzoic acid much the same thing occurred. The drop in urea nitrogen in the latter case, though not so marked, was still suffi-cient (from about 75 to about 50 per cent of the total nitrogen) to show that a substantial part of the urea nitrogen had been used for the synthesis of glycocoll. It is particularly interesting to note here that benzoic acid and phenylacetic acid may be detoxi-cated simultaneously as easily as either of the two singly, or in other words, both glycocoll and glutamine can be made by the body simultaneously as easily as when but one is demanded.

Phenylacetic acid (8) like benzoic acid (9) is detoxicated in the organism of the fowl by a combination of 2 molecules of the acid with 1 molecule of ornithine. The analogy between the two com-pounds, glutamine and ornithine, is too striking to need further comment.

We wished especially to determine the physiological behavior of these detoxication products of phenylacetic acid in organisms other than those in which the original detoxication occurred, for it seemed that these products would either be remodelled into the

detoxication product of phenylacetic acid common to that species of animal or be still further changed chemically in order to reduce the toxicity and facilitate the rapid elimination of the resulting substance in the urine.   Phenylacetyl glutamine was therefore prepared and fed as well as injected into dogs, cats, and chickens. Phenylacetyl ornithine (phenacetornithuric acid) was prepared and fed to dogs and to human beings; and phenylacetyl glycocoll (phenaceturic acid) was prepared and fed to human beings as well as to chickens.

(Phenylacetyl glycocoll)   (Phenylacetyl glutamine)   (Phenylacetyl ornithine)
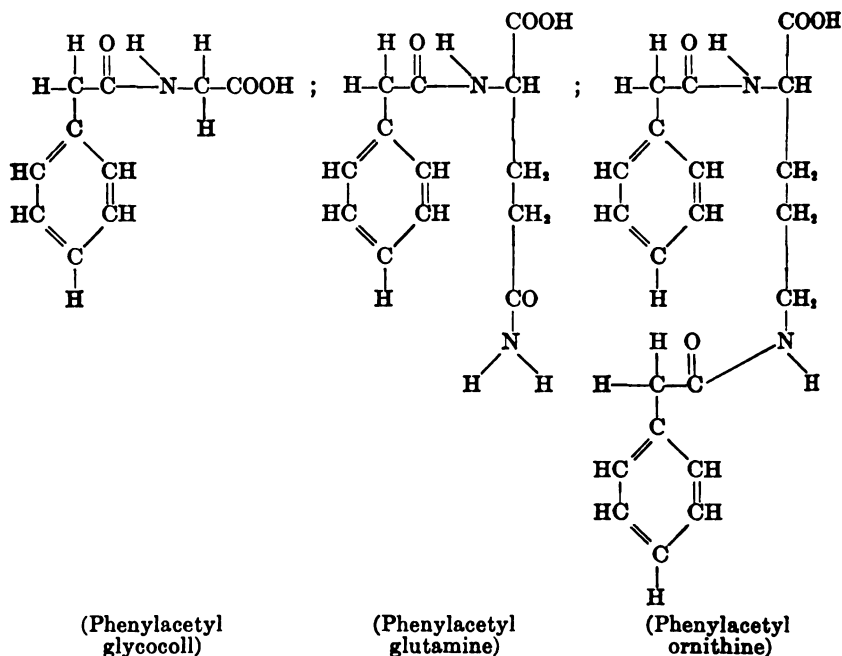
Nearly every protein has yielded on hydrolysis certain quantities of glutamic acid and with this an amount of ammonium salts proportionate to the glutamic acid.   Accordingly, the inference has been drawn that glutamic acid exists in the protein molecule in the form of the amide, *scl.* as glutamine, which has been known for some time to exist in plant proteins.   We, therefore, prepared phenylacetyl glutamic acid both from phenylacetyl glutamine as well as by the Schotten and Baumann synthesis from phenylacetyl

chloride and *d*-glutamic acid. This compound was then fed to human beings in order to determine whether the phenylacetyl glutamine found in the urine of a man after the ingestion of phenylacetic acid could be a secondary product formed from phenylacetyl glutamic acid. The phenylacetyl glutamic acid was also fed to rabbits and to chickens. The phenylacetyl derivatives of asparagine, aspartic acid, alanine, and leucine were fed to dogs, chickens, and to human beings to determine what effect they would have regarding the formation of the detoxication products of phenylacetic acid common to these species.

When a human being was used as the subject in these experiments the substances were ingested as a water solution of the sodium salt. In the cases of the lower animals, including the chickens, the solution of the sodium salt was fed by means of a stomach tube and washed down with plenty of water. When the substances were injected, the exactly neutral sodium salt was prepared and dissolved in isotonic salt solution. When the lower animals were employed as subjects of experimentation, for example dogs, cats, and rabbits, they were placed in metabolism cages. The urine was then collected for a suitable period of time (12, 24, or 36 hours) depending upon the amount of the material ingested. The urine was evaporated to a thick syrup, acidified to Congo red with dilute sulfuric acid, and extracted with a suitable solvent (alcohol, ether, or ethyl acetate) in a rotary extractor of a modified Richter type. The compounds were sometimes crystallized directly from the organic solvent, as in the case of the phenylacetyl glutamine, from the ethyl acetate. In most cases, however, the organic solvent was evaporated to dryness, the residue taken up with water, decolorized with animal charcoal, and recrystallized several times from hot water.

The work on chickens was very much hindered by the great difficulty of separating the urine from the feces. The birds were placed in metabolism cages large enough to allow the free movement of the animal. The most satisfactory flooring material for the cage was found to be a stiff wire net of about ½ inch mesh. This allowed free passage of the urine and the feces into a removeable drawer in the bottom of the cage. The feces and the urine were thus collected together, dried in a current of warm air if necessary, then mixed in a flask with five to ten times their weight

of alcohol.   The flask, with frequent shaking, was then allowed
to stand for at least about 40 hours.   After this time the contents
were filtered and the alcoholic solution was evaporated to dryness
in vacuo or in a current of warm air.   The residue was then ex-
tracted with a small volume of ether to get rid of the fats which
are ever present in the alcoholic extract.   The mass was next
taken up with a small amount of water, acidified to Congo red
with dilute hydrochloric acid, and extracted repeatedly with the
organic solvent which was known to be suitable for the suspected
compound (ether or ethyl acetate was generally used).   In the
preparation of the dibenzoyl ornithine (ornithuric acid) it was
found most profitable simply to place the ether extract in the ice
box for from 1 to 3 weeks and wait until the substance crystallized
out.   In the preparation of the diphenylacetyl ornithine, however,
we found that the process might be hastened by evaporating the
ether solution to dryness, then taking the residue up in alcohol
(in which the material is very soluble), and gradually diluting the
alcoholic solution with water.   The phenacetornithuric acid is
thus forced out of solution in the form of needles when the dilu-
tion is made carefully, and as an amorphous powder when the
water is added in too large amounts.

<div align="center">EXPERIMENTAL.</div>

1. *Phenylacetyl Glycocoll.*

Phenylacetyl glycocoll (phenaceturic acid) was prepared ac-
cording to the Schotten and Baumann reaction by adding to a
glycocoll solution small portions (about ⅓ cc.) of phenylacetyl
chloride together with sufficient sodium hydroxide to keep the
mixture faintly alkaline.   In the meantime the container was
constantly shaken.   The best results were obtained when not less
than 2 molecules of the acid chloride were used for each molecule
of glycocoll.   After all the acid chloride was added the material
was acidified to Congo red with dilute hydrochloric acid and
extracted three times with ether to remove the phenylacetic acid
which was formed during the operation.   The phenaceturic acid
was usually present then as a flocculent, white·precipitate.   This
was then filtered and recrystallized three times from hot water,
after which it was found to be relatively pure.   It melted at 142–

143°C., and according to the Kjeldahl method contained 7.18 per cent nitrogen instead of the theoretical 7.25 per cent.

The substance was weighed out in 5 gm. doses and dissolved in hot water. Sodium hydroxide was then added until neutrality was reached. The solution of the sodium salt thus formed was drunk by a man of 65 kilos body weight who was chosen as the subject of experimentation. The material had no particularly bad taste and proved to be entirely non-toxic. In fact its toxicity was so low that 2 days later the same subject ingested 10 gm. in the course of 24 hours (5 gm. in the morning and 5 gm. in the afternoon of the same day). After a dose of 5 gm., 3.72 gm. of the phenaceturic acid were recovered from the urine, and after a dose of 10 gm., 7.62 gm. were found in the urine. No free phenylacetic acid could be found nor any conjugation product of the same other than the glycocoll compound.

A hen weighing 1.75 kilos was fed on 3 consecutive days 0.5 gm., 1 gm., and 2 gm., respectively, of phenaceturic acid as a solution of the sodium salt by means of a stomach tube. No signs of intoxication appeared. The excreta were treated according to the general plan described above. Of the 3.5 gm. fed, 2.6 gm. of the material were recovered unchanged, but no trace of a compound of ornithine with phenylacetic acid could be found.

2. Diphenylacetyl Ornithine (Phenacetornithuric Acid).

This substance was obtained according to the method of Totani (9) from the excreta of hens after they had been fed phenylacetic acid. The material, though somewhat more easily prepared than its homologue, dibenzoyl ornithine (8), required several weeks to obtain enough for feeding experiments. In our work (10) we have since found that the easiest way to prepare either the benzoyl or the phenylacetyl derivative of ornithine is to place the chickens on a carbohydrate diet for a short time and feed them 1 or 2 gm. of benzoic acid or phenylacetic acid per day. Not only are the hens able to build the ornithine compound very well on a non-protein diet, contrary to the statements found in the literature, but the feces under these conditions are reduced to a minimum and the quantity of urine is increased.

1 gm. of the diphenylacetyl ornithine, in the form of a solution of the sodium salt, was fed to a small dog of 2.7 kilos body weight.

In the urine was found a small amount of phenaceturic acid. No phenylacetyl ornithine, however, could be recovered, probably due, in part at least, to both the small amount of the material ingested as compared with the large amount of solid matter in the urine, as well as to the extreme solubility of the sodium salt of the phenacetornithuric acid. It seemed quite probable, moreover, that the formation of the phenaceturic acid in the organism of the dog was not due to the metabolic alteration of the ornithine into glycocoll, but rather to the splitting of the quite unstable ornithine compound into phenylacetic acid and ornithine, followed by a conjugation of the phenylacetic acid with glycocoll. Accordingly, 1 gm. of the phenacetornithuric acid was converted into the sodium salt, dissolved in isotonic salt solution, and injected subcutaneously into a rabbit. From the urine of the rabbit we were able to recover about 0.2 gm. of phenylacetyl ornithine. Not a trace, however, of the phenaceturic acid could be found. Apparently, therefore, when the compound was not subjected to the digestive processes of the gastrointestinal tract, there was no hydrolysis of the material into its components and consequently no alteration of the compound in the processes of metabolism.

A man ingested two doses of 2 gm. each of phenacetornithuric acid, only the lack of material limiting the size of the dose, for the substance proved to be utterly non-toxic. After the first ingestion of 2 gm. the urine was collected for a period of 12 hours only, evaporated to a thick syrup, acidified, and extracted for 2 hours with absolute ethyl acetate. No crystals of phenylacetyl glutamine appeared on cooling nor on concentration of the extract. It is safe to say, therefore, that no phenylacetyl glutamine was formed. After the ethyl acetate had been evaporated to dryness and the residue had been dissolved in a large volume of ether and allowed to stand in the cold for some time, a very small amount of phenacetornithuric acid (0.1 gm.) crystallized out. After a dose of 2 gm. of the phenacetornithuric acid with a subsequent 24 hour collection of urine, neither phenylacetyl glutamine nor phenylacetyl ornithine could be obtained from the evaporated urine. It is safe to say, however, that the failure to obtain the latter was due to the large amount of solid matter in the residue after so long a urine collection.

3. Phenylacetyl Glutamine.

This substance was prepared according to the method of Thierfelder and Sherwin (11). A man of 62.5 kilos body weight ingested 5 gm. of phenylacetic acid on each of 3 consecutive days. The urine was evaporated to a thick syrup, acidified, and extracted with ethyl acetate. From the ethyl acetate extract was obtained a mixture of phenylacetyl glutamine and phenylacetyl glutamine urea. The mixture of the two substances was then taken up in a saturated solution of barium hydroxide and allowed to stand for a number of hours. In this way the urea was split off from the phenylacetyl glutamine and the barium salt of the latter formed. After standing in the ice box for about 12 hours this solution was treated with carbon dioxide to remove the excess barium. The neutral solution was then evaporated to dryness *in vacuo*, the residue extracted with hot absolute alcohol to remove the urea, the barium salt of the phenylacetyl glutamine dissolved in a small amount of water and acidified with sulfuric acid to remove the barium as barium sulfate, the water solution of the phenylacetyl glutamine extracted with absolute ethyl acetate and the pure phenylacetyl glutamine obtained. That this compound was identical with that originally obtained by Thierfelder and Sherwin is shown by its constants. After drying *in vacuo* at 70°C. the substance melted at 101–104°C., and according to the Kjeldahl method contained 10.49 per cent nitrogen instead of the theoretical 10.69 per cent. The substance was also levorotary.

Phenylacetyl glutamine was fed to a dog in 3 gm. doses as a solution of the sodium salt. A dog of 15 kilos body weight thus received two 3 gm. doses at 12 hour intervals. The urine was collected for 24 hours after the last dose, and from it we obtained 2 gm. of phenylacetyl glutamine and 0.4 gm. of phenaceturic acid. In order to avoid a splitting of the phenylacetyl glutamine in the gastrointestinal tract, the substance was next injected subcutaneously in 1 gm. doses as the sodium salt in isotonic salt solution at 3 hour intervals until 4 gm. in all had been administered. From the subsequent urine we recovered 2.1 gm. of phenylacetyl glutamine (M.P. 99–102°C.), but found no trace of phenaceturic acid.

Phenylacetyl glutamine was fed to a hen in 1 gm. doses as a solution of the sodium salt. The hen received in this way 3 gm.

of the substance in the course of 2 days. The excreta were collected, dried, extracted with alcohol, the alcoholic solution was evaporated to dryness *in vacuo*, taken up with water, acidified to Congo red with dilute hydrochloric acid, and extracted with ethyl acetate to remove the phenylacetyl glutamine. After the separation of the phenylacetyl glutamine from the ethyl acetate, the latter was evaporated to dryness *in vacuo* and the residue extracted repeatedly with ether to remove any diphenylacetyl ornithine. After the feeding of the 3 gm. to the hen, only 0.6 gm. of the original phenylacetyl glutamine was recovered, but no diphenylacetyl ornithine could be found.

4. Phenylacetyl d-Glutamic Acid.

This material was prepared according to the method of Schotten and Baumann by shaking phenylacetyl chloride in excess with a weakly alkaline solution of *d*-glutamic acid (M.P. 203°C.). The same compound was also prepared from phenylacetyl *d*-glutamine by boiling the latter with a saturated solution of barium hydroxide under a reflux condenser until there were no more fumes of ammonia evolved. As this compound of phenylacetic acid is a thick syrup which can be crystallized only with great difficulty, its water solution was employed in all the feeding experiments.

A human being ingested 5 gm. of the substance; 1.5 gm. were fed to a chicken, and 2 gm. to a rabbit. From the urine of the human being 3.2 gm. of the compound were isolated unchanged. From the excreta of the hen 0.45 gm. of the original substance was obtained, and 1.1 gm. of it were recovered from the urine of the rabbit. In no case, however, was any other phenylacetic acid conjugate obtainable.

5. Phenylacetyl Asparagine.

This compound was prepared, like the preceding ones, according to the Schotten and Baumann process. It crystallizes easily, although the yield is very poor. It was identical with the substance previously prepared (12) as shown by the melting point of 180–181°C. after drying at 80°C. *in vacuo*. It contained according to the Kjeldahl method 11.09 per cent nitrogen instead of the theoretical 11.20 per cent.

Phenylacetyl asparagine is apparently somewhat more toxic than the other phenylacetyl derivatives of the amino-acids. A man of 62 kilos body weight ingested 6 gm. of the substance in 2 gm. doses at 24 hour intervals. Each dose was followed by a general feeling of depression and later by severe frontal headaches. From the urine 3.8 gm. of the material were recovered unchanged. After feeding 1 gm. of the compound to a hen in 0.25 gm. doses, 0.2 gm. of it was recovered unchanged, and after the administering of 1 gm. to a rabbit in the same manner, 0.6 gm. of it was recovered from the urine.

6. Phenylacetyl Alanine.

This compound, which had never been studied before, was synthesized as follows: 10 gm. of inactive alanine were dissolved in 50 cc. of water and shaken with about 50 gm. of phenylacetyl chloride (3 molecules of phenylacetyl chloride to 1 molecule of alanine). The acid chloride was added in small amounts with continuous shaking. Sodium hydroxide solution was added in quantities just sufficient to keep the mixture alkaline. The entire process lasted about 2 hours. During the operation it was necessary to stop occasionally and cool the container in ice water to remove the heat of reaction. After the last of the acid chloride had been added, the contents of the bottle were transferred to a 1 liter separatory funnel and acidified to Congo red with dilute sulfuric acid. There appeared at once a heavy, milky mass of solid material. The mixture was then extracted several times with benzene to remove the phenylacetic acid which had formed during the reaction. The white substance which remained after the extraction with benzene, proved by analysis (see below) to be phenylacetyl alanine. The yield from 10 gm. of alanine was about 70 per cent (16 gm.). The compound is fairly soluble in cold water and very soluble in hot water. From the water solution it crystallizes in snow-white, feathery clusters, which when dried at 80°C. melt at 150–152°C. It is quite soluble in ether, ethyl acetate, alcohol, carbon tetrachloride, and *hot* benzene. In the form of a saturated water solution the material was optically inactive. The sodium salt, which is very soluble in water, was found to be also optically inactive. When dried at

80°C. *in vacuo*, the compound showed the following analytical results:

Analysis of Phenylacetyl Alanine.

	Calculated.	Found.
	per cent	*per cent*
C...	63.78	63.77
H...	6.33	6.34
O...	23.17	23.07
N...	6.76	6.34

To form the barium salt of the phenylacetyl alanine, 0.75 gm. of the substance was dissolved in a cold, saturated solution of barium hydroxide. Carbon dioxide was then passed through the solution to remove the excess barium. The barium salt was found to be extremely soluble in cold water, in fact, so soluble that it refused to crystallize even after the solution had been evaporated to a thick syrup.

In order to split the inactive material into its optically active isomers, compounds of the phenylacetyl alanine with brucine, strychnine, quinine, and cinchonine were formed, but all of them were so extremely soluble that it was impossible to obtain any of them in the crystalline form.

We fed 3 gm. of the phenylacetyl alanine in the form of a water solution of the sodium salt to a small dog. The material was apparently physiologically inactive for the animal ate and drank as usual immediately after the feeding. The 24 hour collection of urine was evaporated to a thick syrup. Upon acidification it became almost solid due to the separation of the phenylacetyl alanine. The material was then transferred to a separatory funnel, extracted twice with ethyl acetate, the ethyl acetate evaporated to dryness, and the residue taken up with water from which it was recrystallized. The crystals thus obtained melted sharply at 151–153°C., showing that the phenylacetyl alanine had passed through the organism unchanged, and this almost quantitatively, for of the original 3 gm. that had been fed 2.75 gm. were recovered.

A hen was fed 3 gm. of phenylacetyl alanine in 1 gm. doses on 3 consecutive days. The material was found to be entirely non-

toxic. From the excreta only 1 gm. of the original substance was recovered, but no ornithine compound of phenylacetic acid could be obtained. The substance which was isolated melted at 148–150°C. A human being after ingesting 4 gm. of the phenylacetyl alanine noticed no ill effects. From the urine about 2.5 gm. of the material could be recovered.

7. Phenylacetyl dl-Leucine.

This compound was prepared by dissolving 3 gm. of inactive leucine in 60 cc. of water, placing the solution in a 250 cc. bottle, then adding phenylacetyl chloride in small portions (about 10 gm. in all) with constant shaking, keeping the mixture alkaline the while by the addition of small amounts of sodium hydroxide solution. The entire operation lasted about 2 hours. At the end of this time the contents of the bottle were poured into a 500 cc. separatory funnel and acidified to Congo red with dilute sulfuric acid. Thereupon a mass of white material was thrown out of solution. The acidified mixture was extracted several times with carbon tetrachloride to remove the free phenylacetic acid. The white, flocculent material remaining was then filtered by suction and recrystallized several times from water. When dried at 80°C. *in vacuo* for several hours it melted at 133–134°C. Analysis (see below) proved it to be phenylacetyl leucine. The compound is a white material, crystallizing from hot water in beautiful, feathery clusters. It is very soluble in alcohol, ether, ethyl acetate, and acetone; moderately soluble in benzene; slightly soluble in cold water; but much more easily in hot water. It is just about absolutely insoluble in carbon tetrachloride and petroleum ether. The yield of this and similar syntheses was only about 55 per cent of the theoretical. On analysis, after drying *in vacuo* at 80°C., the following results were obtained:

Analysis of Phenylacetyl Leucine.

	Calculated.	Found.
	per cent	*per cent*
C	67.44	67.49
H	7.68	7.80
O	19.26	18.99
N	5.62	5.72

The sodium, potassium, and ammonium salts of this compound are too soluble to be obtained in the crystalline form. The barium salt is soluble in 25 parts of water and crystallizes in regular and well defined bundles of needles.

The physiological behavior of the phenylacetyl leucine was much the same as that of the other phenylacetylated amino-acids. It was injected intravenously into a rabbit in a dose as large as 1 gm. without evident physiological effects. The original phenylacetyl leucine was recovered afterwards from the urine in an amount which was about 65 per cent of the quantity injected. When 1 gm. of the phenylacetyl leucine was fed to a chicken, the same results were obtained, for from the excreta 0.67 gm. of the substance was recovered. A dose of 4 gm. was taken by a man without causing discomfort. From the evaporated urine 2.27 gm. of the original material were isolated. Apparently it had gone through the organism as the very soluble sodium salt.

<center>DISCUSSION.</center>

The amino group of the α-amino-acids is "blocked" as effectively by the phenylacetyl radical as by the benzoyl radical. In only one instance was there an apparent chemical reaction involving one of the phenylacetylated amino-acids; namely, when diphenylacetyl ornithine was fed to a dog. Here there was evidently an hydrolysis of the compound in the gastrointestinal tract into phenylacetic acid and ornithine, for there was found a small amount of phenaceturic acid in the urine after the feeding but none after the intravenous injection of the material.

It would seem that there is no support to the still extant theory concerning the partial oxidation of benzoylated amino-acids into hippuric acid, nor is there any reason to believe that the amino-acid can enter into chemical reaction of any kind so long as one of the hydrogen atoms of the amino group is replaced by some other radical or element.

At the present time all the evidence seems to indicate that certain amino-acids, namely glycocoll, glutamine, ornithine, and perhaps even cystine, can be built by the animal organism when the subject is in a condition of endogenous protein catabolism, that is, when every avenue to outside protein material or nitrogen of every kind is closed. Furthermore, we know that the nitrogen

for the amino group is obtained from that portion of nitrogen which would otherwise appear in the urea fraction. The problem still confronts us, however, as to the manner in which the amino-acids are catabolized in the animal organism. Are all the different amino-acids normally burned completely into ammonia, carbon dioxide, and water, and is the glycocoll then resynthesized from these end-products, or is glycocoll a common product of the intermediary metabolism of all of these amino-acids? This latter seems rather improbable, since glutamine which contains both an amino and an amide group is synthesized as easily as is glycocoll, and certainly glutamine cannot be considered an intermediary product in the oxidation of any except a very few of the amino-acids at most.

It seems peculiar, to say the least, that there should be apparently three entirely different detoxication processes going on in the organisms of the dog, the hen, and the human being, respectively, when each of the resulting compounds is perfectly harmless when taken into the organism of the other, from which it is easily and rapidly eliminated by way of the urine. At the present time there is no evident explanation of this apparent incongruity. Further investigation of this matter is necessary.

Berczeller (13) has suggested, in connection with the detoxication of foreign organic compounds, that the more important change to be kept in mind is not the chemical alteration of the toxic substance, but rather a change of a purely physical nature. He cites several cases of toxic compounds and compares them with their respective detoxication products. Invariably he finds that there is decidedly a less pronounced lowering of the surface tension of the solution by the detoxication product than by the toxic substance itself. This effect he considers to be the chief aim of the body during the detoxication process. Thus benzoic acid lowers the surface tension of the solution very markedly, while hippuric acid produces a very slight effect in that regard. Again, menthol causes a very decided lessening of the surface tension, while the glycuronic acid compound of menthol has much less effect, and the sodium salt of the latter has practically no such effect at all. Finally, phenol sulfuric acid has only about one-tenth the reducing effect on the surface tension of the solution as has phenol itself. This may be the explanation of the phenom-

enon, it is true, but still undiscovered is the mechanism of the synthetic method by which these amino-acids are prepared in the animal body.

SUMMARY.

Phenylacetyl derivatives of the following natural amino-acids were prepared: glycocoll, alanine, leucine, glutamine, glutamic acid, asparagine, aspartic acid, and ornithine. Of these compounds, phenylacetyl alanine and phenylacetyl leucine were studied in greater detail since their preparation and constants had not been recorded in the literature.

All the above substances were fed to or injected into dogs, rabbits, chickens, and human beings. In every case results showed that when the amino group is phenylacetylated, complete or even partial catabolism of the amino-acid is prevented, thus demonstrating the impossibility of the formation of glycocoll from a more complex amino-acid under these conditions.

Most peculiar and interesting, moreover, is the fact that although different species detoxicate phenylacetic acid according to entirely different reactions which yield completely different compounds, still these products pass unaltered through the organisms of animals other than those in which the original detoxication occurred.

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THE RELATION BETWEEN AGE AND THE CONCENTRA-
TIONS OF PROTEIN FRACTIONS IN THE BLOOD
OF THE CALF AND COW.

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The marked variations in the quantities of certain protein
fractions present in the blood of the new-born calf (1, 2) and the
differences between the blood of young calves and of the adult
cow led us to study the changes which occur in bovine blood with
increasing age. For this purpose we have studied the blood of
three calves from birth to an age of approximately 2 to 3 months.
Samples of blood have been taken from other animals; three heifers
6 months old, three heifers 12 months old, fifteen virgin heifers 17 to
22 months old, and fourteen pregnant heifers 2½ years old. The
heifers 17 to 22 months old were about to be bred and those 30
months old had been pregnant about 3 months. These observa-
tions are supplemented by analyses of the blood of other animals
for a short period (2) and data obtained in studies in another
connection.

Of the calves which were studied continuously one (Calf 669)
received colostrum of a high protein content, a second (Calf 889)
received colostrum which was comparatively poor in globulin,
and a third (Calf 898) did not receive colostrum but was fed
ordinary whole milk from birth. Blood was collected by needle
from the jugular vein and when plasma was obtained coagulation
was prevented by sodium citrate. Blood samples were taken 1
hour after feeding. The calves were fed ordinary whole milk
for 1 month after which they were given small amounts of grain
and hay.

Determinations were made of total nitrogen, fibrin, or fibrinogen
nitrogen, nitrogen content of the protein precipitated by con-

centrations of sodium sulfate of 14.2, 17.4, and 21.5 per cent, and of the non-protein nitrogen. From these determinations values were calculated for fibrin, euglobulin, pseudoglobulins I and II, and albumin, according to the procedures previously

TABLE I.

Data Relating to the Variations in the Blood Proteins of Calf 898 Which Did Not Receive Colostrum soon after Birth. *

Age.	Total N.	"Serum" N	Fibrin N	Euglobulin N.	Pseudoglobulin I N.	Pseudoglobulin II N.	Total globulin exclusive of fibrin.	Albumin N.	Non-protein N
New-born.	0.636	0.581	0.055	0.016	0.028	0.144	0.189	0.322	(0.070)
days 1	0.659	0.602	0.057	0.008	0.050	0.144	0.202	0.330	(0.070)
2	0.703	0.604	0.099	0.002	0.052	0.139	0.183	0.351	(0.070)
3	0.758	0.636	0.122	0.000	0.046	0.159	0.205	0.361	(0.070)
4	0.757	0.594	0.163	0.045	0.025	0.120	0.190	0.359	0.045
6	0.862	0.656	0.206	0.047	0.046	0.157	0.240	0.379	0.037
8	0.901	0.668	0.233	0.008	0.060	0.126	0.194	0.437	0.037
14	0.957	0.763	0.194	0.023	0.051	0.124	0.198	0.511	0.054
20	0.887	0.759	0.138	0.029	0.012	0.153	0.194	0.511	0.054
27	0.854	0.740	0.114	0.037	0.049	0.107	0.183	0.516	0.041
34	1.024	0.804	0.220	0.048	0.069	0.149	0.260	0.503	0.041
41	0.977	0.876	0.101	0.035	0.120	0.111	0.266	0.560	0.050
48	0.974	0.944	0.030	0.095	0.101	0.113	0.309	0.577	0.058
55	0.961	0.887	0.074	0.037	0.132	0.103	0.272	0.570	0.045
62	0.998	0.935	0.063	0.072	0.113	0.165	0.350	0.541	0.045
69	0.895	0.841	0.054	0.049	0.095	0.099	0.243	0.553	0.045
76	0.891	0.847	0.054	0.051	0.099	0.074	0.224	0.590	0.033
83	0.875	0.828	0.047	0.052	0.104	0.084	0.240	0.548	0.040
90	0.918	0.841	0.077	0.063	0.100	0.088	0.261	0.544	0.036
97	0.959	0.862	0.097	0.066	0.088	0.136	0.290	0.532	0.040
111	0.981	0.908	0.073	0.049	0.114	0.124	0.287	0.573	0.048

* Results are expressed as grams of nitrogen in 100 cc. of blood plasma or serum.

outlined (3). The data are contained in Tables I to VI and Chart 1. The relation between the corpuscles and plasma was determined by measuring the volume of each after centrifuging at a constant speed. The average proportions of corpuscles and plasma for Calves 898 and 899 were 38 to 62 and 44 to 55, respectively. These relations held throughout the experiment.

In the chart the data from each of the calves (Calves 669,[1] 899, 898), have been plotted for each of the blood constituents estimated with the exception of the total plasma nitrogen and the non-protein nitrogen. In place of the total plasma nitrogen,

TABLE II.

*Data Relating to the Variations in the Blood Proteins of Calf 899 Which Received Colostrum Relatively Low in Globulin.**

Age.	Total N.	"Serum" N.	Fibrin N.	Euglobulin N.	Pseudoglobulin I N.	Pseudoglobulin II N.	Total globulin exclusive of fibrin.	Albumin N.	Non-protein N.
Colostrum.	1.916	Casein=1.122	0.258	0.181	0.095			0.101	0.062
New-born.	0.685	0.623	0.062	0.033		0.186	0.219	0.363	0.041
days									
1	0.877	0.764	0.093	0.116	0.204	0.136	0.451	0.276	0.038
2	0.949	0.808	0.141	0.128	0.151	0.145	0.424	0.335	0.049
3	0.950	0.853	0.097	0.115	0.185	0.174	0.474	0.334	0.045
4	1.006	0.866	0.140	0.132	0.198	0.144	0.474	0.347	0.048
5	0.848	0.726	0.122	0.073	0.146	0.166	0.375	0.306	0.045
8	0.927	0.792	0.135	0.083	0.144	0.132	0.359	0.392	0.041
12	0.935	0.852	0.083	0.136	0.141	0.152	0.429	0.367	0.054
18	0.878	0.794	0.084	0.072	0.124	0.103	0.299	0.450	0.045
25	0.901	0.833	0.068	0.078	0.093	0.140	0.311	0.483	0.041
32	0.947	0.883	0.064	0.095	0.083	0.165	0.343	0.495	0.045
39	0.882	0.824	0.058	0.043	0.083	0.100	0.226	0.548	0.050
46	0.955	0.899	0.056	0.072	0.087	0.142	0.301	0.540	0.053
53	0.864	0.827	0.037	0.054	0.100	0.133	0.287	0.482	0.053
60	0.923	0.842	0.081	0.075	0.093	0.097	0.265	0.532	0.045
67	0.856	0.784	0.072	0.050	0.087	0.127	0.264	0.475	0.045
68	0.858	0.796	0.062	0.083	0.084	0.104	0.272	0.487	0.037

* Results are expressed as grams of nitrogen in 100 cc. of blood plasma or serum.

values for serum nitrogen obtained from the analysis of the filtrates from the precipitation of fibrin or fibrinogen have been recorded. These results were taken for comparison because the fibrinogen of blood appears to vary somewhat independently of the other blood constituents.

[1] Data relating to the absorption and disappearance of agglutinins of Calf 669 have been presented in another connection (4).

From an inspection of the tables and of the chart the following points are evident:

Serum Nitrogen.—During the first weeks of life the quantity of serum nitrogen present in the blood of young calves depends

TABLE III.

*Data Relating to the Variations in the Blood Proteins of Calf 669 Which Received Colostrum Relatively High in Globulin.**

Age.	Total N.	"Serum" N.	Fibrin N.	Euglobulin N.	Pseudoglobulin I N.	Pseudoglobulin II N.	Total globulin exclusive of fibrin.	Albumin N.	Non-protein N.
New-born.		0.768		0.039	0.051	0.175	0.265	0.422	(0.081)
2hrs. 40mins.		0.768		0.000	0.128	0.177	0.282	0.405	(0.081)
5 " 40 "		0.960		0.133	0.264	0.162	0.559	0.319	0.081
19 " 40 "		1.126		0.307	0.333	0.158	0.798	0.290	0.038
days									
3		1.160		0.298	0.320	0.133	0.751	0.358	0.051
13		1.186		0.299	0.141	0.111	0.551	0.558	0.077
21		1.071		0.239	0.128	0.073	0.440	0.597	0.034
31		0.972		0.064	0.174	0.069	0.307	0.622	0.043
41		1.005		0.045	0.196	0.071	0.312	0.667	0.026
50		0.994		0.062	0.115	0.115	0.294	0.666	0.034
57		0.959		0.051	0.098	0.102	0.251	0.661	0.047
65		0.937		0.033	0.189	0.077	0.289	0.601	0.047
71		0.948		0.082	0.120	0.098	0.289	0.616	0.043
78		0.917		0.030	0.106	0.107	0.233	0.653	0.021
85		0.937		0.042	0.058	0.087	0.187	0.713	0.037
92		0.931		0.036	0.111	0.085	0.232	0.658	0.041
106		1:032		0.071	0.093	0.173	0.337	0.646	0.049
113		0.959		0.072	0.091	0.153	0.319	0.584	0.049
118		1.038		0.081	0.114	0.113	0.308	0.681	0.049

* Results are expressed as grams of nitrogen in 100 cc. of blood plasma or serum.

upon the quantitative nature of the diet just after birth. If the calf does not receive colostrum but is fed ordinary milk the serum nitrogen increases gradually for about 6 weeks. This increase is due, essentially, to the increase in albumin. In case colostrum has been ingested the serum nitrogen values indicate the effect of the absorption of globulins from the colostrum. After approxi-

TABLE IV.

Data Relating to the Proteins in the Blood of Calves 6 and 12 Months Old.

Age.	Total N.	"Serum" N.	Fibrin N.	Euglobulin N.	Pseudoglobulin I N.	Pseudoglobulin II N.	Total globulin exclusive of fibrin.	Albumin N.	Non-protein N.
mos.									
	1.098	1.027	0.071	0.124	0.210	0.132	0.466	0.516	0.045
6	1.082	0.991	0.091	0.104	0.223	0.120	0.447	0.494	0.050
	1.030	1.002	0.028	0.129	0.178	0.140	0.437	0.507	0.058
Average....	1.070	1.007	0.063	0.116	0.203	0.131	0.450	0.506	0.051
	1.087	1.038	0.049	0.077	0.206	0.136	0.419	0.547	0.062
12	1.074	1.006	0.068	0.070	0.210	0.145	0.425	0.523	0.058
	1.059	1.021	0.028	0.097	0.210	0.161	0.468	0.503	0.050
Average....	1.073	1.022	0.048	0.081	0.208	0.137	0.437	0.524	0.057

TABLE V.

Data Relating to the Proteins in the Blood of Different Non-Pregnant Heifers 18 to 22 Months Old.*

Age.	Total N.	"Serum" N.	Fibrin N.	Euglobulin N.	Pseudoglobulin I N.	Pseudoglobulin II N.	Total globulin exclusive of fibrin.	Albumin N.	Non-protein N.
mos.									
17	1.132	1.027	0.105	0.083	0.202	0.156	0.441	0.516	0.070
18	1.219	1.043	0.176	0.115	0.260	0.120	0.495	0.478	0.070
18	1.359	1.270	0.089	0.181	0.355	0.126	0.672	0.520	0.078
18	1.104	1.002	0.102	0.049	0.256	0.103	0.408	0.524	0.070
18	1.060	0.978	0.082	0.034	0.235	0.091	0.363	0.545	0.070
19	1.185	1.130	0.055	0.070	0.256	0.152	0.478	0.582	0.070
19	1.082	1.002	0.080	0.062	0.231	0.136	0.429	0.515	0.058
20	1.099	1.047	0.052	0.090	0.239	0.145	0.474	0.515	0.058
20	1.305	1.126	0.179	0.042	0.395	0.157	0.594	0.462	0.070
20	1.261	1.134	0.127	0.057	0.380	0.132	0.569	0.511	0.054
20	1.316	1.114	0.202	0.042	0.375	0.140	0.557	0.499	0.058
21	1.175	1.035	0.140	0.078	0.279	0.138	0.495	0.490	0.050
22	1.168	1.097	0.071	0.035	0.353	0.132	0.520	0.515	0.062
22	1.183	1.048	0.135	0.058	0.301	0.136	0.495	0.483	0.070
22	1.184	1.084	0.100	0.036	0.293	0.136	0.464	0.557	0.062
Average...	1.188	1.073	0.115	0.069	0.296	0.133	0.494	0.514	0.064

* Results are expressed as grams of nitrogen in 100 cc. of blood plasma or serum.

mately 6 weeks serum nitrogen fluctuates somewhat but is lower than the concentration found in the adult animal or in the calves 6 months old.   The adult values appear to be attained between 3 and 6 months of age.

Albumin Nitrogen.—Changes in the albumin nitrogen are more or less independent of the variations in the other proteins.   The

TABLE VI.

Data Relating to the Proteins in the Blood of Different Pregnant Heifers 30 Months Old.

Age.	Total N.	"Serum" N.	Fibrin N.	Euglobulin N.	Pseudoglobulin I N.	Pseudoglobulin II N.	Total globulin exclusive of fibrin.	Albumin N.	Non-protein N.
mos.									
	1.079	1.015	0.064	0.099	0.260	0.112	0.471	0.482	0.062
	1.052	0.964	0.088	0.061	0.214	0.136	0.411	0.491	0.062
	1.142	1.083	0.059	0.072	0.293	0.128	0.493	0.528	0.062
	1.043	0.957	0.086	0.116	0.251	0.136	0.503	0.396	0.058
	1.129	1.070	0.059	0.101	0.268	0.120	0.489	0.523	0.058
	1.085	1.033	0.052	0.072	0.297	0.148	0.517	0.458	0.058
30	1.064	1.010	0.054	0.129	0.198	0.099	0.416	0.532	0.062
	1.095	1.047	0.048	0.086	0.243	0.120	0.449	0.540	0.058
	1.225	1.136	0.089	0.092	0.364	0.140	0.596	0.478	0.062
	1.123	1.085	0.038	0.194	0.256	0.132	0.582	0.441	0.062
	1.133	1.074	0.059	0.158	0.207	0.144	0.509	0.499	0.066
	1.090	1.021	0.069	0.130	0.177	0.161	0.468	0.487	0.066
	1.150	1.070	0.080	0.196	0.288	0.096	0.580	0.423	0.066
	1.163	1.113	0.050	0.189	0.285	0.136	0.610	0.437	(0.066)
Average....	1.112	1.048	0.064	0.121	0.252	0.132	0.506	0.479	0.062

value at birth is slightly higher than on the following day. From this time there is a gradual increase up to approximately 3 weeks, after which there is a tendency toward a slight increase with irregular fluctuations.   The important fact to be brought out with regard to the albumin nitrogen is that changes in the concentration of albumin, particularly in the first 3 weeks of life, do not appear to be correlated with the changes in the concentration of the globulins.

Fibrin Nitrogen.—The data on fibrin nitrogen are in part, in the case of Calves 898 and 899 and of the older animals, based on duplicate determinations by two different procedures; coagulation following recalcification with calcium chloride and precipitation with 10.6 per cent sodium sulfate. The first procedure represents fibrin and the second fibrinogen. With the quantities of plasma used the results by the two methods usually agree.[2]

From the data on Calves 898 and 899 it might appear that there is a higher fibrinogen content of the blood early in life, during the period when adjustments in the other proteins are taking place. The determinations of fibrin on various young animals indicate a considerable individual variation and no relation of age to the fibrinogen concentration of the blood. A consideration of data on the adult animals also tends to substantiate this idea. It is evident that the variations in the concentration of fibrinogen of the calves is not directly related to the variations in the other serum proteins. The daily records on Calves 898 and 899 do not lend much assistance in interpreting the variations in the fibrinogen content of the blood. Calf 898 had a temperature approximately 1° higher than Calf 899 at the time the fibrinogen content of its blood was rising but during the period of high fibrinogen values the temperature was approximately that of Calf 898. From the work of Smith and Little (5) on the effect of colostrum upon the new-born animal, it is probable that this calf was conducting a battle against an infection which was not present in the case of Calf 899. The large increase in fibrinogen at the age of 34 days in the case of Calf 898 occurred at the same time as an abraised and swollen knee-joint which was not present the period before this one and had disappeared before the time of the next analysis. Calf 899 does not present any marked temperature changes. In the latter part of the experiment he was subject to a deranged digestive apparatus due to the ingestion of binding twine in his bedding which caused partial occlusion of the rumen and interfered with his normal metabolic activities. The data obtained during the last month of life for this calf are open to question as far as it may be considered as representing a normal animal.

[2] Unpublished data.

Days 0 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 112 119 126 133 140 147 154 161

CHART 1.

486
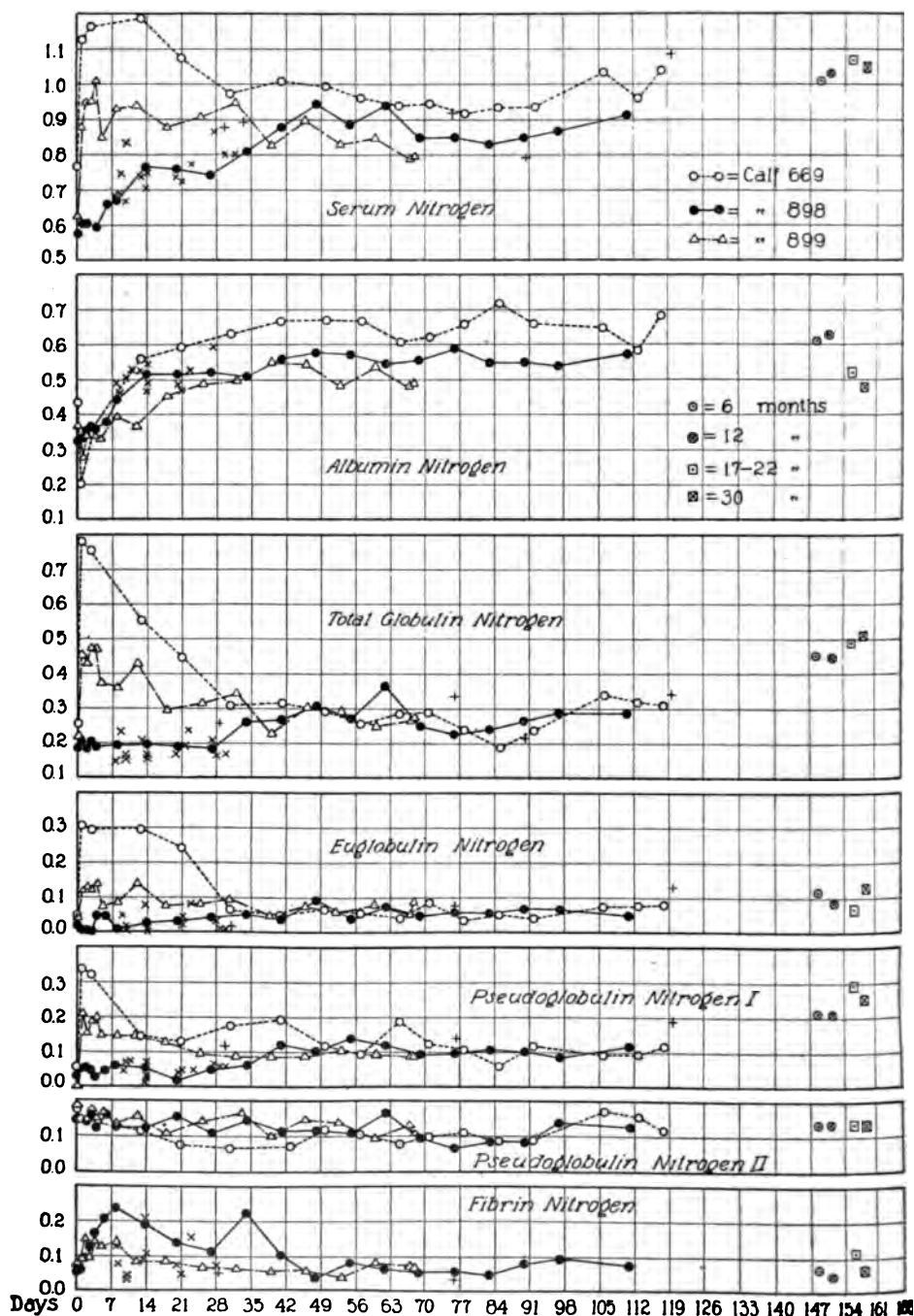
CHART 1. Curves showing the variations in various protein fractions of blood with increasing age. Calf 898 did not receive colostrum, Calf 899 received colostrum relatively low in globulins, and Calf 669 received colostrum rich in globulins. The crosses (X) indicate results obtained on calves which did not receive colostrum; some of these animals were injected with cow serum, some were injected and fed cow serum, and some were fed cow serum (5). The results on these animals agree essentially with those obtained on animals which had not received colostrum or which would have received milk containing very small amounts of globulins. The plus signs (+) indicate results on animals which had received colostrum soon after birth. Average data for calves 6 and 12 months of age, non-pregnant heifers 17 to 22 months old, and pregnant heifers 30 months old are plotted as indicated in the legend. The data are presented in terms of grams of nitrogen in 100 cc. of blood plasma or serum.

The work of Foster and Whipple (6) indicates that cell injury, inflammation, intoxication, or liver injury, will affect the formation of fibrin. If we admit the probable systemic infection (5), then the general high fibrin values for Calf 898 and the increase in fibrin at the time of the swollen knee-joint might be explained by the findings of these investigators.

Total Globulin.—In the case of Calf 898 which did not receive colostrum the values for total globulin nitrogen remain practically constant for 4 weeks after which they increase to values which are essentially the same as those for calves which received colostrum. At about 10 weeks there appears to be a tendency for the total globulin to decrease slightly. At 6 months the total globulin values approach those of the adult animal. The two calves which received colostrum showed a marked increase in globulin during the first day of life after which the total globulin shows a tendency to decrease during a period of from 3 to 4 weeks. Following this change the amount of total globulins is approximately the same regardless of the previous diet. The concentration of globulin present during the first 3 to 4 weeks of life is directly related to the quantity of globulin absorbed during the first days of life. Colostrum ingested by Calf 669 was very thick and rich in globulin whereas the colostrum ingested by Calf 899 was relatively poor in globulin.

Euglobulin.—Remarks with regard to the ingestion of colostrum and the variation in protein nitrogen relating to the total globulin apply to euglobulin. At birth there is essentially no euglobulin present. When euglobulin is not obtained from the colostrum or otherwise it appears in the blood gradually and at approximately 5 to 6 weeks reaches the values found in calves which received colostrum. The euglobulin content of the adult animal is variable, due to influences which have not been determined. The data relating to euglobulin in the case of Calf 898 show a certain quantity of euglobulin during the first week of life. These values are probably only in a degree accurate and are due in part to technical error. The reason for this assumption is, that in the case of serum at such ages there is not a visible precipitation at 14.2 per cent of sodium sulfate.

Pseudoglobulin I.—In the first weeks of life the pseudoglobulin I content of the blood is related to the nature of the diet im-

mediately after birth. At an age of approximately 5 to 6 weeks the values are the same no matter what the diet may have been. The adult animal has a much higher pseudoglobulin I content than the calf 3 months old.

Pseudoglobulin II.—The quantity of protein precipitated, between 17.4 and 21.5 per cent of sodium sulfate, appears to be independent of the diet and is practically constant for all ages. This condition exists in spite of considerable fluctuations in the protein content of the blood on either side of this fraction.

Animals 6 to 30 Months of Age.—Data relating to the concentration of the various protein fractions in animals 6 to 30 months of age are contained in Tables IV to VI. The average values are indicated in Chart 1. The blood samples for calves 6 and 12 months old were collected at the same time and under similar feeding conditions. The samples from the virgin heifers and the pregnant heifers were collected about 2 weeks apart. The general conditions of stabling and of feeding were essentially the same; the animals were still on winter feed and had not been pastured. The results of the analyses indicate certain minor individual variations in the distribution of the proteins of the blood. The effect of age is not particularly apparent after the calves are 6 months old. The only indication of a difference between the two groups of adult animals is in the euglobulin fraction which is higher in the non-pregnant heifers than in the pregnant heifers. On the other hand, the concentrations of fibrin are in general higher in the virgin heifers than in the pregnant heifers. This is rather surprising since, from the work of Fahræus (7) on human serum there is a greater suspension stability in pregnant than in non-pregnant women. The suspension stability appears to be in part at least related to the fibrinogen and globulin fractions of the plasma, in which fibrinogen has a greater individual effect than the other globulins. Our observations do not necessarily contradict those of Fahræus for we are dealing with a different organism and we have not made determinations of suspension stability.

A calculation of the relative proportions of globulin nitrogen and albumin nitrogen to the total nitrogen gives for virgin heifers 49 per cent of the total serum protein as globulin and 51 per cent as albumin; for the pregnant heifers the values are 51 per cent of

total globulin nitrogen and 49 per cent of albumin nitrogen. These values differ from those of Robertson (8, 9) who found 36 per cent of total globulins and 64 per cent of total albumins for the ox.   Robertson's average values for Hammarsten's (10) determinations of ox serum are 58 per cent total globulin and 42 per cent total albumin. We have found a number of cases in which the serum of the adult animal contained a preponderance of globulin over the albumin but only a few animals, except in the case of calves, in which the albumins predominated over the globulins.   We are dealing, of course, with the cow while Robertson may have been studying the steer; we do not have any evidence relating to sex.

DISCUSSION.

Studies of the variations in the distribution of proteins with increasing age are comparatively few. The most detailed investigations are those of C. E. Wells (11) on the rabbit and of Toyama (12) on the albino rat.   Toyama's investigation followed that of Hatai (13) on the total protein content of the serum of the same animal.   Reiss (14) and Utheim (15) have made observations on infants.   These investigators have all determined the proteins by means of the refractometer.   In the work on rabbits and rats the procedure of Robertson (9) for the separation of proteins has been used.   Lewis and H. G. Wells (16) have recently presented some analyses of human blood using the method employed in this work.

It is necessary to be very cautious in comparing results obtained upon different species of animals.   The work of Robertson (9) has brought out species, as well as individual, differences in the proportions of albumin and globulin in the serum of the rat, rabbit, horse, and ox.   A difference between the infant and the calf is shown in the data of Lewis and Wells.   These investigators confirm to a certain extent for the infant our observations on calves; they found that blood obtained from the umbilical cord of infants does not contain euglobulin.   On the other hand, their data indicate quantities of pseudoglobulin I in the blood of infants comparable to those present in adult man, whereas in calves this protein is essentially absent.

The most extended series of analyses of the total protein of the blood of infants and children has been made by Utheim (15) who confirms the work of Reiss. It was found that the concentration of total protein remains practically constant at 6 to 6.5 per cent from birth to about the 10th to 11th month when it begins to rise. The adult level is reached at about the 15th month. The serum of premature infants contains less protein, 4.5 per cent, than that of infants born at full term. The normal level for infants is not attained until about 3 months of age.

Alder (17) has studied the blood of man by means of the refractometer and viscosimeter and finds practically no difference between men and women; little difference in the composition of blood serum (a) between the ages of 7 and 70 years, (b) as a result of the ingestion of food, (c) following muscular activity, (d) between venous and capillary blood, and (e) from day to day. Placental blood contained, as a rule, less protein and a higher proportion of albumin than adult blood. The percentage of total protein in placental blood, 5.7 to 7.0 per cent, is higher than that for new-born calves, 3.6 to 5.6 per cent. Most of the values for calves lie between 3.6 and 4.8 per cent; only two samples out of twenty-eight showed a value above 4.8 per cent, the average is 4.4 per cent. The results of Lewis and Wells on placental blood, 4.3 to 6.7 per cent (N $\times$ 6.25), agree in general with those of Alder.

From the consideration of published data it is apparent that the blood of a new-born animal has a lower total protein content than that of the adult animal and that during the early part of life there is an increase in the total protein concentration of the serum. In infants and rats the protein content of the serum the first days after birth appears to be slightly lower than at birth or a few days later. With rats (13) there is a rapid increase in protein up to the time of weaning at which time the protein content shows some irregularity. The increase then continues until sexual maturity is attained when there is again an irregularity followed by further slight increases to the adult level. In the case of rabbits (11) and infants (13, 14) the available data indicate a gradual increase in total protein from birth to maturity.

In the studies just reviewed the effect of the nature of the diet immediately following birth has not been considered. The data

presented in this paper indicate that the quantity of total protein present in the serum of calves is definitely related to the quantitative composition of the colostrum or milk ingested soon after birth. The *quantitative* variations in the composition of blood plasma imposed by the absorption of the proteins of colostrum are transient. At the age of from 4 to 6 weeks, the quantitative effect of the absorbed protein has practically disappeared and the composition in the blood serum tends to become the same no matter what the previous diet may.have been. These remarks apply to the normal animal.

The relative distribution of the various protein fractions of blood serum with increasing age has been studied extensively in but two cases, Wells on rabbits and Toyama on white rats. Wells did not find any "correspondence between the ages of the animals and the variations of the relative proportions of 'insoluble' globulin, 'soluble' globulin and albumins" in the blood serum of the rabbit. His youngest animal was 21 days old. The data of Toyama relate to the rat from birth to maturity. He found a gradual increase in quantity of globulin and albumin present in the blood serum, which was most rapid in the suckling period, 23 days. Immediately following the suckling period, at 30 days, there was a fall in the quantity of globulin but not in the albumin, while at the next age studied, 50 days, the albumin values dropped slightly while the globulin showed an increase.

The data presented on calves and cows indicate that during approximately 4 to 6 weeks of life the proportions of the different protein fractions precipitated from the blood by sodium sulfate are affected by the diet of the calf soon after birth; *i.e.*, by the character of the milk ingested. Following this period the absolute and relative proportions of globulins are approximately the same. The proportions of globulins characteristic of the adult animal are attained at the age of from 18 to 22 months. When colostrum containing euglobulin and pseudoglobulin I is fed there is a rapid absorption of protein by the calf (1). The absorbed globulins then gradually disappear, in part at least. When globulins are not absorbed they are formed gradually and attain values similar to those which occur following the ingestion of colostrum at about the time when the absorbed globulins have fallen to the average values for a calf 4 to 6 weeks old. The variations in globulin content just

discussed relate particularly to those globulins precipitated by concentrations of sodium sulfate less than 17.4 per cent, euglobulin and pseudoglobulin I. Pseudoglobulin II remains relatively constant at all times. This constancy of the pseudoglobulin II fraction suggests that the variations in the other proteins with feeding and age are true variations in protein and not the secondary effect of changes in the water content of the blood; *i.e.*, that the water content is adjusted to changes in the protein content of the blood.

The albumin concentration of calf serum is low at birth and rises rather rapidly during the first 2 weeks, at the end of which time the adult level is approached. Variations in the concentrations of albumin are apparently not affected by the ingestion of colostrum; essentially the same conditions exist whether or not globulin is absorbed. The non-protein nitrogen appears to be higher at birth, then to decrease slightly, and to rise again to the adult value, which approaches that at birth.

The proportions of albumin and globulin in non-pregnant and pregnant heifers are approximately equal. This statement applies to separations made with sodium sulfate. We have reason to believe that similar results would be obtained with ammonium sulfate and possibly a slightly higher proportion of globulin would be indicated when magnesium sulfate is used as the total globulin precipitant. The most marked difference between the adult blood and that of a calf 3 months old is in the proportion of pseudoglobulin I present in the blood. It appears that one of the chief adjustments between these ages is in the pseudoglobulin I fraction. The data which we have presented as indicating the composition of adult blood apply to females which have just reached maturity. Certain analyses indicate that the blood of older animals may vary widely, particularly in the fibrinogen, euglobulin, and pseudoglobulin I fractions. Some animals have a relatively high euglobulin concentration.

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THE RATE OF HYDROLYSIS OF WHEAT GLIADIN.*

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Braconnot in 1820 was the first to hydrolyze proteins with acid. He boiled meat as well as glue with sulfuric acid and isolated leucine and glycocoll from the resulting mixtures. These were the first amino-acids obtained from protein substances.

Alkalies were also used as hydrolyzing agents at an early date, for Mulder (1839) obtained leucine from meat which had been boiled with sodium hydroxide.

The view that proteins are complex compounds built up of amino-acids originated with Liebig, but the manner in which amino-acids are linked with each other in the protein molecule has only been established in recent years. In 1902 Hofmeister thoroughly examined and discussed the various ways in which one might imagine two amino-acids could be united with each other in the protein molecule, and pointed out that the only linkage which was entirely probable was that which involved the scheme,

$$R - NH - CO - R$$
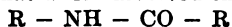
This linkage has been termed by Fischer the peptide bond. The actual existence of this configuration in the protein molecule has been established by the work of Fischer and Abderhalden and their associates, who have isolated numerous polypeptides from partially hydrolyzed proteins. But

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whether or not all the amino-acids are united in peptide union in the protein molecule, is a question that cannot be answered by work of this nature.

The peptide bond is split on hydrolysis with the formation of an amino group and a carboxyl group. Consequently, if the peptide bond occurs extensively in the protein molecule, the products of hydrolysis should contain large amounts of free carboxyl and amino groups. The isolation of much of the nitrogen of proteins in the form of amino groups of amino-acids renders it highly probable that the amino-acids actually are united in the protein molecule in peptide union, but proof of this can only be obtained when the progress of hydrolysis has been shown to be accompanied by the formation of free amino and carboxyl groups.

It is obvious that a study of the progress of hydrolysis of proteins is dependent úpon the development of an analytical method whereby either free amino or free carboxyl groups may be estimated in the hydrolysis mixture. The earliest purely chemical method for doing this is due to Siegfried (1905) who found that amino-acids would unite in the cold with calcium hydroxide and carbon dioxide to form calcium salts of carbamino-acids. On heating, these decompose and an amount of calcium carbonate precipitates which is equivalent to the amount of amino nitrogen present. Mathieu (1909) made use of this property to follow the rate of hydrolysis of gelatin by boiling 1.55 M acid, but without very satisfactory results.

Sörensen in 1908 introduced the formol titration method whereby the amount of carboxyl liberated by the hydrolysis of peptide bonds could be measured. This method was used by Henriques and Gjaldbäk in 1910 to ascertain the conditions under which proteins could be hydrolyzed completely, and in 1911 to follow the enzymatic hydrolysis of several proteins. It is evident from their results that after the protein is completely hydrolyzed the solution contains large amounts of free amino and carboxyl groups and also, that the process of hydrolysis actually is accompanied by the formation of increasing amounts of these two groups. Although Henriques and Gjaldbäk apparently did not appreciate it themselves, this was the first definite chemical evidence that the amino-acids were for the most part united in the protein molecule by means of the peptide bond.

Van Slyke in 1911 introduced his nitrous acid method to estimate free amino nitrogen and the application of this method to the products of protein hydrolysis by numerous investigators has served further to verify the views of Hofmeister and Fischer.

Nasse in 1872 clearly pointed out that the nitrogen which gives rise to ammonia on hydrolysis of proteins must be bound differently in the molecule from the nitrogen which is found as amino groups of amino-acids after hydrolysis. Since that time Nasse's "loosely bound nitrogen" has been variously termed "amide nitrogen" or "ammonia nitrogen," but no very clear evidence that the ammonia was derived from an amide group in the protein molecule was presented until 1908, when Osborne, Leavenworth, and Brautlecht pointed out the close correspondence between the amount of ammonia required by theory for amide formation with one of the carboxyl groups of the aspartic and glutaminic acids in numerous proteins, and the

amount of ammonia actually obtained from them after acid hydrolysis. Further evidence was obtained by Thierfelder and von Cramm (1919) who found the proportions of ammonia removed by a definite mild hydrolysis from synthetic polypeptides containing glutamine to be almost identical with the proportion removed from gliadin under the same conditions. Furthermore, Osborne and Nolan (1920) demonstrated the appearance of an acidity in the solution when gliadin was hydrolyzed by dilute acid, which was closely equivalent to the amount of ammonia liberated by the hydrolysis, and which was best explained by the hypothesis that the ammonia was derived from amides of the dicarboxylic acids.

Dakin's (1918) discovery of oxyglutaminic acid, which he found in casein, gliadin, and glutenin, and which was found by Jones and Johns (1921) in lactalbumin, has raised a question as to the value of conclusions regarding the origin of ammonia from amide nitrogen based upon the amounts of glutaminic and aspartic acids obtained from proteins. Nevertheless, it seems almost certain that the large amounts of ammonia obtained from most proteins must be derived from the hydrolysis of amides of these dicarboxylic acids.

The discovery of the basic amino-acids, of tryptophane, and of proline, in proteins has shown that in addition to the simple peptide bond and the grouping which gives rise to the ammonia, nitrogen occurs in a guanidino group (arginine), in an imidazole group (histidine), and in an indole ring (tryptophane) as well as in the pyrollidine ring of proline and oxyproline. Moreover, the small amount of free amino nitrogen found only in those proteins which contain lysine is probably due to the end-standing amino group of this amino-acid. These nitrogenous systems are all, save tryptophane, stable to acid, but the guanidino group of arginine is quite readily decomposed by alkalies, breaking down to form carbon dioxide and ammonia. Tryptophane is also unstable to alkalies.

When methods involving the measurement of amino nitrogen are used to determine the rate at which a protein is hydrolyzed, account must be taken of the observation of Fischer and Abderhalden (1904), that proline can enter into polypeptide union not only with its carboxyl group, but also with its imino nitrogen group. A union of this latter type if existing in a protein would be hydrolyzed without the formation of an amino group and would hence escape detection. There is no method at present available to detect imino nitrogen peptide union if it occurs in the protein molecule.

In addition to the types of union which have been definitely proved to occur in proteins there are other types which possibly may exist in them. Andersen and Roed-Müller (1915) have presented evidence which indicates the possible presence of very small amounts of uramino-acids, although substances containing the uramino group or derivatives of it, have never been isolated from proteins. Johnson and Burnham (1911) have drawn attention to the possibility that sulfur may occur in proteins in thiopeptide, $-CS - NH -$, union which implies the existence of nitrogen in a grouping different from any of those mentioned.

From these considerations it is apparent that a study of the rate of hydrolysis of proteins is limited to the study of the rate at which, on the one hand, ammonia appears in the solution, and on the other hand, the rate at which peptide bonds are broken with the formation of amino groups. Such a study shows only the rate of decomposition of the hypothetical amide group and the rate of hydrolysis of the peptide bonds, exclusive of those in which the imino group of proline plays a part.

The rate at which proteins are hyrolyzed by *acids or alkalies* has received very little study since the introduction of suitable analytical methods, although much attention has been paid to the rate of hydrolysis by enzymes.

Van Slyke (1911) made use of his nitrous acid method to estimate amino nitrogen to study the rate of hydrolysis of egg albumin by 5 per cent sodium hydroxide at 60°, but his experiments were carried out mainly with the object of demonstrating the applicability of the method.

Pittom in 1914 used Sörensen's formol titration to follow the rate of hydrolysis of casein and egg albumin when boiled with 20 per cent hydrochloric acid. He also determined the rate at which ammonia is set free from these proteins as well as the rate at which substances precipitable by phosphotungstic acid are hydrolyzed, and showed that each of these reactions has a very high velocity in the early stages of the hydrolysis. The ammonia was practically all set free within an hour while the amino nitrogen rapidly increased in amount during the first 2 or 3 hours, the rate of hydrolysis thereafter being much slower.

Harding and Fort (1918) followed the rate of hydrolysis of the protein of the human placenta by boiling 20 per cent hydrochloric acid, using Van Slyke's method.

The rate of hydrolysis of proteins by enzymes has been studied by Henriques and Gjaldbäk (1911) using Sörensen's method; Walters (1912, *a* and *b*), who worked with casein and precipitated the unaltered casein by neutralization and then estimated the nitrogen in the precipitate; Andersen (1915) also using Sörensen's method, who determined how far hydrolysis could be carried by enzymes alone; and by Frankel (1916), Northrop (1919), and Dunn and Lewis (1921) who followed the rate of enzymatic hydrolysis of numerous proteins by means of Van Slyke's method.

We have studied the rate at which ammonia is set free from gliadin by various concentrations of hydrochloric acid ranging from 0.1 to 20 per cent at boiling temperature, at the same time observing the rate of hydrolysis of the peptide bonds as indicated by the appearance of free amino nitrogen in the solution. In addition we have carried out a few series of experiments with sulfuric acid, as well as with sodium and barium hydroxides, in order to ascertain the effect upon the rate of hydrolysis of a less highly ionized acid and of alkaline reagents.

Gliadin was selected for our experiments since it can be readily obtained in a state of purity, it has been thoroughly investigated with regard to its amino-acid make-up, and moreover, it contains a larger proportion of amide nitrogen, glutaminic acid, and proline than any other protein hitherto analyzed.

Osborne and Nolan (1920) observed that 1 per cent hydrochloric acid liberates almost as much ammonia from gliadin on boiling for 2 hours as is set free by 20 per cent hydrochloric acid on boiling for 24 hours. We have found that ammonia is set free from gliadin quite rapidly at first by boiling 0.027 N (0.1 per cent) hydrochloric acid. Under the experimental conditions adopted, the neutralization of the acid by the ammonia set free is, in this case, the deciding factor in the rate of hydrolysis. Nevertheless, hydrolysis slowly proceeded until when the solution had been boiled for 118 hours, over 90 per cent of the free acid had been thus removed.

By boiling with 0.1 N hydrochloric acid nearly all the ammonia is set free from gliadin in 11 hours, while with 0.2 N acid the same point is reached in 5 hours. The rate of liberation of ammonia by acids more concentrated than 1.0 N is so rapid in the early stages as to render measurement impossible by the methods used, but with these higher concentrations of acid another phenomenon becomes evident. It has long been known that the total amount of ammonia obtainable from a protein could be slightly increased by prolonged hydrolysis with concentrated acids (Denis, 1910). This is due to secondary decomposition and much of the additional amount of ammonia probably originates from the decomposition of tryptophane and cystine. The tables of data for the rate of hydrolysis of amide nitrogen by 1.0 N and more concentrated hydrochloric acid show this slow secondary decomposition very clearly.

0.2 N sulfuric acid is less effective as a hydrolyzing agent than 0.2 N hydrochloric acid, the curve lying between those showing the rate of hydrolysis by 0.1 and 0.2 N hydrochloric acid for most of its length (Chart I).

With alkaline hydrolyzing reagents secondary decomposition, especially that of arginine, contributes a much larger proportion of the total ammonia than is the case with acid reagents. The rate of hydrolysis of the amide nitrogen appears to be even more

rapid with 0.2 N sodium hydroxide than with 0.2 N hydrochloric acid. The rate at which the ammonia is set free shows that.three distinct phases of the reaction exist. The greater part of the ammonia is liberated rapidly and this phase represents the hydrolysis of the amide nitrogen. When all the amide nitrogen is set free, ammonia comes off at a much slower but quite steady rate for a number of hours. This phase probably represents the decomposition of arginine. Finally when an amount of nitrogen has been liberated corresponding roughly with the known amount of amide nitrogen and one-half the arginine nitrogen, the rate of liberation of ammonia becomes exceedingly slow. This final phase probably represents the decomposition of amino-acids other than arginine. All of these reactions probably proceed simultaneously but become evident on the curve as they are successively terminated.

0.2 N barium hydroxide is a more rapid hydrolyzing agent than 0.2 N sodium hydroxide with respect to the amide nitrogen but causes secondary decomposition at a slower rate (Chart II).

By hydrolyzing gliadin with dilute acid it is possible to remove nearly all of the amide nitrogen and at the same time split very few of the peptide bonds. It is therefore possible to secure products from gliadin which still retain a large proportion of the peptide bindings unbroken and which are practically free from amide nitrogen. The study of these products will form the subject of a later paper.

While our experiments with 1.0 and 2.0 N acid were not prolonged until hydrolysis had been entirely completed, we have no reason to doubt that it would be possible to push the hydrolysis, at any rate, very nearly to completion. Whether or not weaker acid reagents can eventually split all the peptide bonds at boiling temperature is not certain.

Hydrolysis of gliadin as measured by the appearance of amino nitrogen is practically complete when the protein has been boiled about 20 hours with 20 per cent hydrochloric acid or for about 50 hours with 4 N hydrochloric acid.

The curves which show the rate of hydrolysis of gliadin by the stronger acid-hydrolyzing reagents have no irregularities, but indicate that if hydrolysis is sufficiently prolonged it will eventually become complete. The peptide bonds of the protein are

broken successively in a perfectly smooth manner, the process continuing until the entire molecule is hydrolyzed to amino-acids. The action of acid-hydrolyzing agents is thus in sharp contrast to that of enzymes; pepsin, for example, as has been shown by the work of Frankel (1916), hydrolyzes a protein to a certain point at which action ceases. It thus is evident that there are portions of the protein molecule which are resistant to the action of this enzyme. Precisely the same remarks apply to the action of trypsin, but it is clear that they cannot be applied to the hydrolytic action of strong acids on the protein. A fundamental difference, therefore, exists between the hydrolysis of a protein by acids on the one hand, and by enzymes on the other hand (Chart III).

The initial rate at which the peptide bonds of gliadin are split by alkaline reagents is much more rapid than the initial rate at which they are split by acids of equivalent concentration. Apparently there are certain peptide bonds in the protein molecule which are extremely susceptible to attack by alkali. These break up very rapidly and the process then continues at a rate resembling that observed when equivalent concentrations of acid are used.

The rate of hydrolysis effected by barium hydroxide is much more rapid than that by an equivalent concentration of sodium hydroxide. No explanation of this observation can be suggested but it appears that the stability of the peptide bond is in some way influenced by the presence of the divalent metallic ion (Chart IV).

Gliadin was prepared according to the directions of Osborne and Harris (1906) with the exception that no sodium chloride was added to the water used for precipitating the concentrated alcoholic solution. In the air-dry condition this preparation analyzed as follows:

	per cent
Nitrogen	16.03
Moisture	8.64
Ash	0.18
Nitrogen, ash- and mosture-free	17.58

The hydrolysing reagents were made up of such a concentration that when 20 cc. of gliadin solution (1 gm.) were added to 80 cc. of reagent the concentration would be exactly 0.1 N, 0.2 N, etc.

The procedure was as follows: 80 cc. portions of hydrolyzing reagent were pipetted into 200 cc. Pyrex flasks and warmed under a reflux condenser on an electric hot-plate to about 90°C.   20 cc. of 5 per cent gliadin solution in 70 per cent alcohol, of which the concentration was controlled by nitrogen determinations, were then run in.   By this means a solution of 1 gm. of gliadin in 100 cc. of 0.1 N, 0.2 N, etc., hydrolyzing reagent was conveniently obtained and the hydrolysis begun, from a definite point of time.

At the end of the desired period of boiling the contents of the flasks were rapidly cooled and treated with sodium hydroxide until a precipitate which separated during the addition of the alkali had just redissolved.   At this point the solutions were slightly acid to phenolphthalein.   Magnesium oxide was then added in excess and the ammonia distilled into tenth normal acid.   The distillation was contined until about 60 to 70 cc. of residue remained.   This was made up to 100 cc. and aliquots of 10 cc. were withdrawn, after careful shaking, for amino nitrogen determinations by the Van Slyke method.   Total nitrogen was also determined in a 25 cc. aliquot to serve as a check against the ammonia determination.

When alkaline hydrolyzing agents were used the procedure was somewhat modified.   An adapter on the upper end of the reflux condenser was dipped into a flask containing dilute sulfuric acid to catch the ammonia. The hydrolysis was carried out in 750 cc. flasks and at the end of the hydrolysis period sufficient acid was run in through a dropping funnel to neutralize the contents.   The condenser was then adjusted to the distilling position without disconnecting at any point and water and excess of magnesia were added to the flask.   Distillation of the ammonia could thus be effected without loss.   It was found impossible to titrate the ammonia obtained by alkaline hydrolysis of gliadin on account of foul smelling decomposition products in the distillate which rendered the end-point uncertain.   Moreover, hydrogen sulfide was found in the distillate.   The nitrogen in the distillate was therefore determined by the Kjeldahl method.

All determinations were made in duplicate.

During the hydrolysis of gliadin by acids of different concentrations certain precipitation and color reactions occurred which require mention.

With 0.1 N hydrochloric acid there was no distinct separation at any period of the hydrolysis although the solution became opalescent.   With 0.2 N and higher concentrations of acid a precipitate began to appear soon after boiling had begun, the more rapidly, the higher the concentration of the acid up to 4.0 N. This precipitate slowly dissolved as hydrolysis proceeded, 16 or more hours being necessary with 0.5 N acid but only about 3 hours with 4.0 N hydrochloric acid.

There was no marked color change save a slight darkening of the yellow solution, on long hydrolysis with any acid less concentrated than 2.0 N.

With 2.0 N hydrochloric acid after 65 hours boiling the solution had become pale green, while with 4.0 N acid a green color appeared in the clear solution after 5 hours boiling and in 16 hours the solution was emerald green.

With 20 per cent hydrochloric acid there was no precipitation but the pink Liebermann reaction developed a few minutes after adding the protein to the hot acid. This color soon changed to yellow-brown and in 4 hours the solution was distinctly green. Longer periods of hydrolysis changed the color to brown but no separation of black humus occurred.

The changes observed when 0.2 N sulfuric acid was used resemble those noted with 0.1 N hydrochloric acid and those with 4.0 N sulfuric acid were very similar to the changes observed with 2.0 N hydrochloric acid.

No precipitation of protein material or color changes were observed during hydrolysis with alkalies, the color remaining yellow throughout. Considerable inorganic material derived from the flasks was observed especially when alkaline hydrolysis was prolonged.

The temperature at which the hydrolyses occurred was influenced by the presence of the alcohol and ranged from 93 to 96° with the weaker hydrochloric acid reagents. Loss of alcohol, probably through the formation of ethyl chloride, occurred with hydrochloric acid stronger than 2.0 N and the temperature consequently rose on prolonged hydrolysis.

The non-appearance of humus in the solutions which had been hydrolyzed by 20 per cent hydrochloric acid is due to the small concentration of protein in the solution. When 2 gm. or more of gliadin were boiled in 100 cc. of 20 per cent hydrochloric acid, sufficient humus was formed to appear as a precipitate.

Tables I to XIII give the percentage of the total nitrogen of gliadin found as ammonia nitrogen and the percentage of the total available amino nitrogen found as amino nitrogen after hydrolysis as noted. The total available amino nitrogen of gliadin was taken as 57.3 per cent of the total nitrogen (Van Slyke, 1912). This figure was confirmed by an estimation of the total amino nitrogen obtained from a sample of the gliadin used in this work.

TABLE I.

0.027 N HCl at 93–94°C.

Time.	Total N as ammonia N.
hrs.	*per cent*
5	7.7
15.5	13.2
20	14.3
28	15.5
44	16.8
70	17.7
118	19.5

TABLE II.

0.1 N HCl at 93–94°C.

Time.	Total N as ammonia N:	Total amino N.
hrs.	*per cent*	*per cent*
1	8.5	
2	14.4	3.7
3	17.4	
4	18.8	3.5
8	21.7	5.3
11	22.9	6.3
17	23.4	7.6
26	24.1	13.6
40	24.2	17.3

TABLE III.

0.2 N HCl at 93–94°C.

Time.	Total N as ammonia N.	Total amino N.
hrs.	*per cent*	*per cent*
0.5	10.7	
1	14.0	2.5
3.5	21.8	4.6
6	23.8	
16	23.6	15.6
24	23.9	23.3
40	24.4	30.0

TABLE IV.

0.5 N HCl at 93–94°C.

Time.	Total N as ammonia N.	Total amino N.
hrs.	*per cent*	*per cent*
0.5	17.4	
1	21.0	4.3
2	23.4	6.5
4	24.5	12.3
16	24.6	29.7
22	24.9	37.9
24	24.9	40.2
40	25.0	56.8

TABLE V.

1.0 N HCl at 94–95°C.

Time.	Total N as ammonia N.	Total amino N.
hrs.	*per cent*	*per cent*
0.5	22.2	
1	23.6	9.6
2	24.2	11.5
7	25.0	31.2
12	24.8	42.8
14	24.9	46.7
16	25.0	49.2
18	24.9	51.1
22	25.0	57.5
27	25.0	59.9
40	25.1	69.1
49	25.3	76.9
69		83.7

TABLE VI.

2.0 N HCl at 94–96°C.

Time.	Total N as ammonia N.	Total amino N.
hrs.	*per cent*	*per cent*
1	24.7	21.7
3.3	24.9	39.5
5	25.1	43.7
7	24.9	50.2
9	25.0	55.5
12	25.2	62.1
17	25.5	74.7
24	25.4	79.5
45	25.2	84.0
65	25.5	88.5

TABLE VII.

4.0 N HCl at 98–104°C.

Time.	Total N as ammonia N.	Total amino N.
hrs.	per cent	per cent
0.5	24.9	30.4
1	24.8	45.0
2.5	25.3	56.6
4.5		70.8
5	25.4	75.0
8		83.5
13	25.4	89.3
16		90.0
24	25.5	95.3
40		96.9
48	25.5	98.2
72	25.5	98.5

TABLE VIII.

20 per cent HCl at 102–110°C.

Time.	Total N as ammonia N.	Total amino N.
hrs.	per cent	per cent
1	25.2	59.2
2		71.5
3	25.2	80.1
4	25.3	82.1
5	25.4	88.2
7.5	25.5	91.8
11	25.5	95.9
15	25.4	97.6
19		98.7
40	25.6	98.8

TABLE IX.

0.2 N H₂SO₄ at 93–94°C.

Time.	Total N as ammonia N.	Total amino N.
hrs.	per cent	per cent
1	9.4	
2	14.8	
5	21.6	
6	22.6	
12	23.3	4.9
16	24.4	
24	24.7	
48	25.2	22.1
71	25.6	29.8

TABLE X.

4.0 N H₂SO₄ at 96–98°C.

Time.	Total N as ammonia N.	Total amino N.
hrs.	*per cent*	*per cent*
1	25.0	21.5
3	25.1	40.6
5	25.2	46.9
7		56.2
11	25.3	64.9
17	25.6	76.9
24	25.5	82.0
41	25.5	86.3
65	25.6	94.0

TABLE XI.

0.2 N NaOH at 93–94°C.

Time.	Total N as ammonia N.	Total amino N.
hrs.	*per cent*	*per cent*
1	17.8	8.4
2	22.7	10.1
3	24.7	
4	25.5	13.4
5	26.4	14.6
6	27.2	17.2
8	29.0	18.1
12	29.2	
17		20.5
46	29.9	26.2
72		31.8

TABLE XII.

1.0 N NaOH at 94–95°C.

Time.	Total N as ammonia N.	Total amino N.
hrs.	*per cent*	*per cent*
1	26.8	17.1
2	28.1	24.0
3	28.5	28.9
5	29.2	33.4
6		34.6
8	30.0	39.0
15		47.3
24	29.8	60.3

TABLE XIII.

0.2 N Ba (OH)₂ at 93–94°C.

Time.	Total N as ammonia N.	Total amino N.
hrs.	per cent	per cent
1	23.7	15.0
3	26.3	21.8
5	26.7	26.7
7		29.3
8	27.1	
17	29.0	39.3
46	30.1	56.9
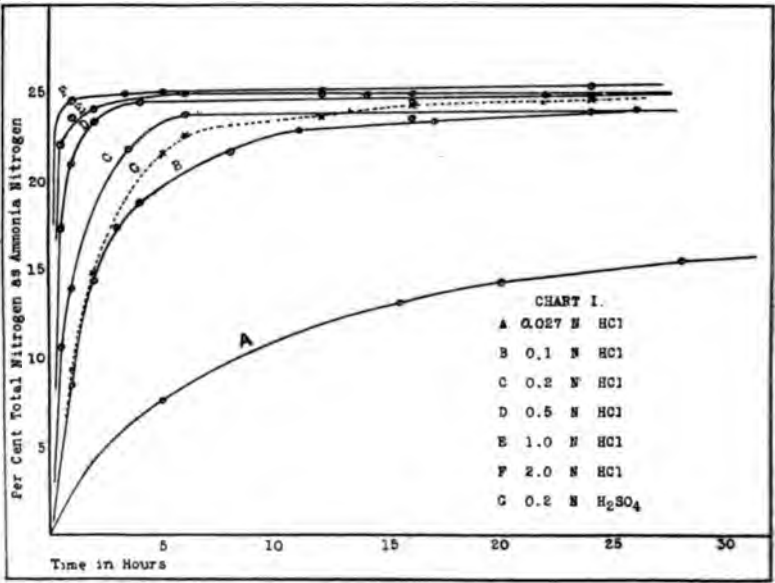
CHART I. Amide hydrolysis of gliadin by acids.
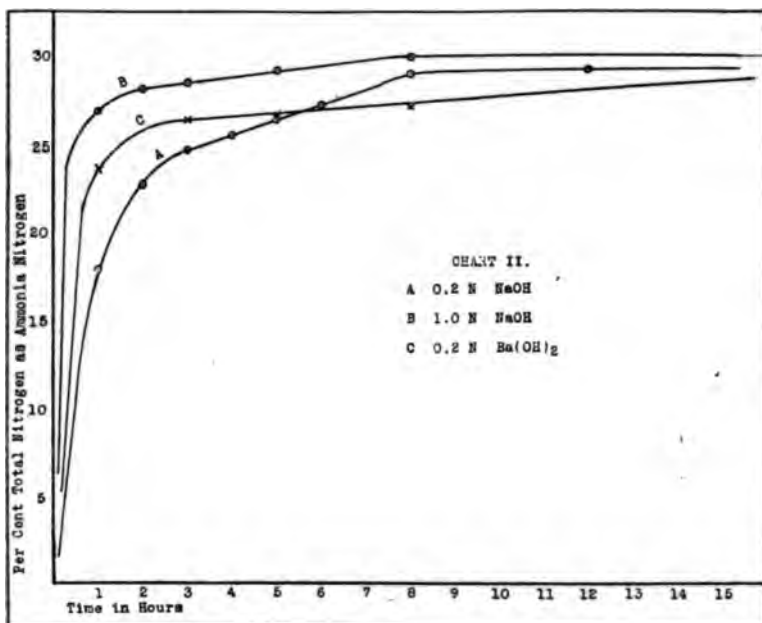
CHART II. Amide hydrolysis of gliadin by alkalies.
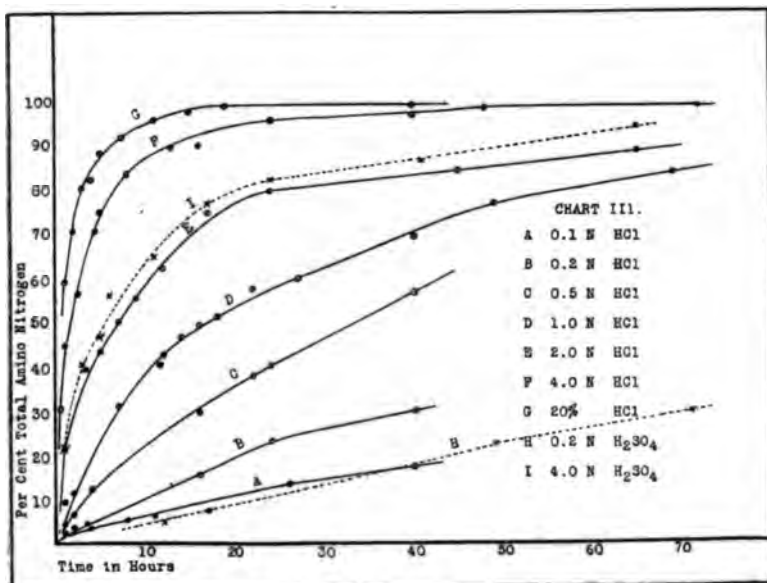
CHART III. Peptide hydrolysis of gliadin by acids.
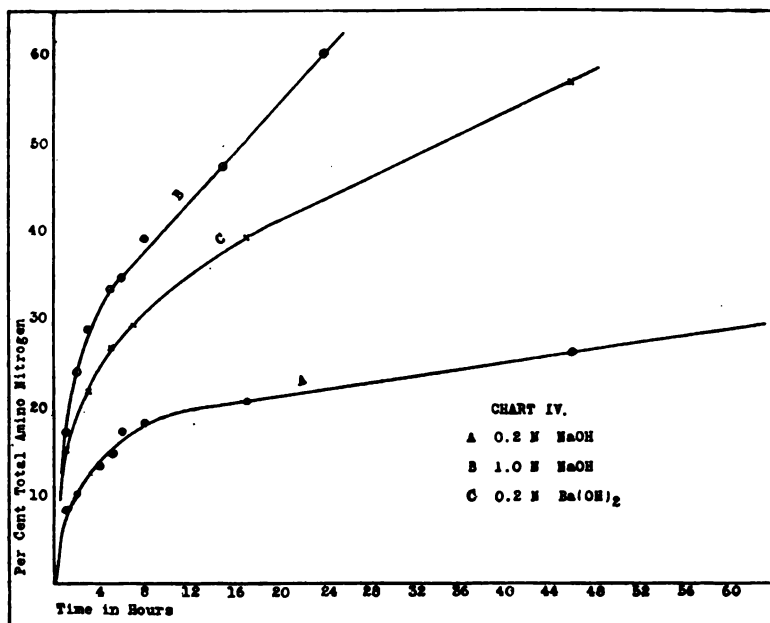
CHART IV. Peptide hydrolysis of gliadin by alkalies.

SUMMARY.

1. The rate at which gliadin is hydrolyzed at boiling temperature by the following reagents has been investigated: 0.027, 0.1, 0.2, 0.5, 1.0, 2.0, 4.0 N hydrochloric acid; 20 per cent hydrochloric acid; 0.2, 4.0 N sulfuric acid; 0.2, 1.0 N sodium hydroxide; 0.2 N barium hydroxide.

2. The liberation of ammonia from gliadin, presumably amide hydrolysis, is readily effected at boiling temperature by very dilute acid or alkali. The ammonia is set free with great rapidity by the stronger acid reagents.

3. Peptide hydrolysis is nearly completed by boiling gliadin with 20 per cent hydrochloric acid for 20 hours or with 4 N hydrochloric acid for 50 hours. Much longer periods are required when less concentrated acid reagents are used.

4. Alkalies hydrolyze gliadin more rapidly in the early stages of hydrolysis than equivalent concentrations of acids in respect to both amide and peptide bindings. Due to secondary decom-

position, the amount of ammonia set free by alkalies is considerably greater than that liberated by acid reagents.

5. Barium hydroxide hydrolyzes gliadin more rapidly than sodium hydroxide of equivalent concentration in respect to both amide and peptide bindings.

6. By the use of varying concentrations of acid-hydrolyzing reagents a picture of the hydrolysis of gliadin has been obtained from the splitting of the first bonds to the completion of the reaction. Acid hydrolysis is thus shown to be a continuous process proceeding from first to last without marked interruption due to the existence of stable complexes, and is therefore clearly distinguished from enzymatic hydrolysis.

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ON ABNORMAL MILK AND ON THE INFLUENCE OF AN ASEPTIC UDDER INFLAMMATION ON THE COMPOSITION OF THE MILK.

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In 1921 we analyzed a number of abnormal milks which possessed the chemical composition that is considered to be characteristic for milk from udders with streptococcic infections.

In several of these samples streptococci were absent. The number of leucocytes in nearly all, with or without streptococci, was very high.

These results gave us the impression that the rôle usually ascribed to these bacteria in producing udder inflammations and the secretion of abnormal milk, is exaggerated. We examined this point by producing a sterile inflammation of one of the quarters of a normal udder and by comparing the milk of the thus treated quarter with that of the normal quarters as well as with the milk of the quarter before the inflammation. For this purpose a solution of 0.2 per cent silver nitrate was under sterile conditions injected into one quarter (R. F.) of a healthy milch cow in full lactation.

We also used a second way to find out whether the streptococci are as important in causing abnormal milk as is usually believed. This second method consisted in the analyzing of milks of udder-diseased cows brought to the abattoir and by making a bacteriological and anatomical examination of the udder tissue.[1]

The interesting paper of Baker and Breed (1) supported our opinion that the importance of the streptococci is not so great as is generally believed. Notwithstanding this fact, they concluded

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[1] We are indebted to Mr. H. S. Frenkel for the examination of the udder tissues.

that the abnormalities of their samples of milk were due to strepto-cocci. We believe that another interpretation is not excluded. From eleven samples with very high pH values (6.92 to 7.0) they found one free from streptococci. Again in four out of ten samples with high pH values (6.84 to 6.92) these bacteria were absent. The same result obtained with five samples out of thirteen with pH values between 6.76 and 6.84.

We cannot see the impossibility of the abnormality of these samples being caused by any other agent than streptococci.

Methods.

We generally used the methods of the Dutch Codex Alimen-tarius for milk (3rd edition). The titratable acidity was deter-mined with 0.1 or 0.25 N NaOH, using phenolphthalein as indi-cator and expressing the acidity in the number of cc. of 0.25 N NaOH used for 100 cc. of milk.

The oxalate acidity was estimated in the same way after adding 4 cc. of a neutral 10 per cent solution of potassium oxalate per 25 cc. of milk.

We think it worth while to point out the fact that milk of abnormal acidity is detected easily by adding a phenol red solution to the oxalated milk, as normal milk gives the transition tint of phenol red.

The chlorides were estimated by adding nitric acid to the milk, filtering, and using the Volhard method in the filtrate as described in the above mentioned Codex.

For lactose we usually chose the method of Folin and Denis.[2] For very abnormal samples this method cannot be used, as a persistent blue-violet color appears during the boiling.

We then applied the iodometric titration (of the excess of $CuSO_4$) after boiling with Fehling's solution. For the estimation of the total protein we used the Kjeldahl method. The casein was precipitated with a saturated solution of potassium-aluminium sulfate. With abnormal milks neither this method nor the other methods recommended for the elimination of casein, give exact results. The calcium was determined in the protein-free fil-trate (trichloroacetic acid was used to remove the proteins) by pre-

[2] Folin, O., and Denis, W., *J. Biol. Chem.*, 1918, xxxiii, 521.

cipitating the faintly acid solution (indicator methyl orange) with ammonium oxalate, contrifuging, washing, and titrating with $KMnO_4$ solution.

For the carbonic acid estimation we used the apparatus of D. D. Van Slyke, acidifying with lactic acid. As recommended by Van Slyke and Baker[3] the CO_2 was absorbed with a solution of NaOH. The tryptophane was determined colorimetrically by the method given by Fürth and Nobel (2) by means of the reaction of Voisenet. The number of leucocytes was estimated by the method of Baker and Breed (3).

For the sediment estimation we used the well known Trommsdorff tubes for 10 cc. of milk with capillair. In 1921 we used for the determination of the non-protein nitrogen metaphosphoric acid; in 1922 we precipitated the proteins with sodium tungstate and sulfuric acid (Folin and Wu). The filtrate was nesslerized after destruction. The pH values were found with the indicator method as described by Baker and Van Slyke (4). Instead of brom-cresol purple we used phenol red as we preferred the latter. We must remark that the indicator method does not give exact figures with very abnormal milks nor with colostrum. These milks are usually colored.

DATA AND DISCUSSION.

Table I gives the data of the milk samples from the milch cow which was treated with silver nitrate solution. The right fore quarter was injected on March 8. The samples Nos. 381 R. F. and L. F. were taken March 9, about 14 and 24 hours after injection. The milk from the right fore quarter was abnormal both times, as is seen from the data of the table.

Again the following day the composition and appearance of the milk from the injected quarter were abnormal, whereas the other quarters gave normal milk. Gradually the abnormality of the milk diminished; for instance, the composition of the milk of March 16, though still abnormal, differed less from normal milk than that of the previous days. The appearance of this milk was almost normal. The quantity of milk drawn from this quarter, which was very small in the first days after the injection,

[3] Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1919, xl, 335.

TABLE I.

Milk from a Cow Which Was Treated with Silver Nitrate Solution and with Turpentine.

The R.F. quarter injected with silver nitrate on Mar. 8.

Nos. 381a, 382a, 383a, 385b, 386a, 387a, and 388a were drawn from the quarter injected with silver nitrate.

Nos. 337, 343, and 375 were taken before the injection.

Nos. 381b, 382b, 383b, 385a, 385c, 386b, 387b, and 388b were taken from other quarters than that injected with silver nitrate.

Nos. 349, 351, 358, and 363 were taken some days after the injection with turpentine.

Date.	No.	Quarter.	Acidity.	Oxalate acidity.	pH	Sediment.	Leucocytes.	Non-protein nitrogen.	Chlorides.	Lactose.	Total proteins.	Casein.	CaO	CO₂ in 2 cc. of milk.	Tryptophane in serum.
						per cent		*mg. per 100 cc.*	*mg. per 100 cc.*	*per cent*	*per cent*	*per cent*	*mg. per 100 cc.*		*mg. per 100 cc.*
Mar. 9	381a	R.F.	2.5			0.35	Somewhat too many.	53.3				2.7	120.0		109.6
" 9	382a	R.F.	3.3			2.2			298.4		8.05	3.8	152.0	0.477	348.0
" 10	383a	R.F.	3.7			5.0									300.0
" 13	385b	R.F.	5.3	1.3	6.98–7.06	Very much.	Too many to count.		280.0						171.0
" 14	386a	R.F.	5.35			4.0					5.75	3.1	199.7	0.55	90.0
" 16	387a	R.F.	4.8			0.5				2.7					
" 17	388a	R.F.	4.8	0.0	6.75–6.82				153.6		3.53	2.6	196.4		
Jan. 3	337*	4 quarters.	7.0			0.25	Normal.	28.8	104.0		2.39				Normal.
" 11	343*	4 "	7.6	2.2		0.3	"	32.9	90.9	4.43	2.92		245.0		"
Feb. 24	375	R.F.	8.6	4.25		0.35	"		88.4	3.8					
Mar. 9	381b	L.F.	7.0			0.1	"								

Date	No.									
Mar. 9	382b	R.H., L.F., L.H.								
" 10	383b	R.H.	8.6	4.2	6.5–6.6	0.6	Many.			84.3
" 13	385a	L.F., L.H.	8.86	4.6						
" 13	385c	R.H.	8.24			0.25	Normal.			
" 14	386b	R.H.					Somewhat too many.			
" 16	387b	R.H.	8.0			0.2	Normal.			
" 17	388b	R.H.	5.8							
Jan. 14	349†	4 quarters.	8.4	3.2		0.25	Normal.			106.6
" 16	351†	4 "	10.3	4.5		0.3	"			96.6
" 24	358	4 "	8.2			0.4	"	235.0		79.8	22.0
" 31	363	4 "				0.25	"	205.7	0.13	72.2	21.0

* The milk of the 4 quarters was analyzed separately. The differences were of no importance.
† The sediment was determined separately in the 4 quarters with the same result.

had largely increased. However, on March 19 and later, it was impossible to get any milk from this quarter notwithstanding the fact that it had been milked regularly at the same hours as the other quarters. We could never find streptococci in the milk of the treated quarter.

The figures of Table I show that the milk of this quarter resembles milk of quarters with streptococcic infection. This will be confirmed by some of the data given in Table II. It applies to different constituents: chlorides, lactose, total proteins, carbonic acid, calcium, and tryptophane, and holds good also for the acidity, pH values, number of leucocytes, and sediment. The non-protein nitrogen, estimated twice, was once too high. The milk of the other quarters had normal appearance, acidity, and pH value; also the content of the chlorides, number of leucocytes, and amount of sediment were normal; with the exception, that the sediment of the R. H. quarter was on March 10 a little too high.

The low acidity of the milk of the R. F. quarter was to be expected, as the composition of milk from inflamed udders approaches that of blood plasma (5). Nevertheless, we have found in several milks from quarters with streptococcic infections high as well as low acidities. The high acidity in these samples may be caused partly by the acid formation of these bacteria and partly by a high percentage of globulins, as in colostrum.

Later on we will discuss the tryptophane figures. Our experiment proves that other affections than streptococcic invasions can produce the same changes in milk. Therefore, aseptic and bacteriological inflammations have in general the same influence on the chemical composition of milk. Our results make it more probable that streptococcic infections of the udder are usually originated by non-bacteriological lesions; namely, that the streptococcic invasion is secondary.

Table I also gives the figures of some samples of milk of the same cow taken before the treatment with silver nitrate. There is no reason to discuss these figures as they are normal. The same cow was used 2 months earlier for another experiment; *i.e.*, to examine whether an aseptic abscess in other parts of the body than the udder, in this case in the region of the neck, influences the composition of the milk. The abscess was produced by sterile injection of turpentine. It developed slowly to a

rather large size. Neither during the development nor afterwards was the composition of the milk much affected. Only once did the milk of one of the quarters give a sediment that was a trifle too high (it was 0.6 per cent). Sometimes the acidity was too high; *i.e.*, 10.3 (it was determined within half an hour after the drawing). Instead of 90 to 100 mg. we found in some of the samples 80 and 72 mg. of Cl. The milk with 72 mg. of Cl contained 0.14 cc. of CO_2 in 2 cc. Once (4 days after the injection with turpentine), we found in the milk a great many corpuscles (cells) with round nuclei of different sizes.

Concerning the results formerly (in 1912) found in this laboratory in milk of cows suffering from different diseases, we mention merely that disturbances of the digestion and external diseases frequently influenced the composition of the milk (6). Chlorides and total proteins were increased, lactose and acidity decreased.

The alcohol test, *i.e.* mixing the milk with an equal volume of 70 per cent alcohol, often gave a positive result; namely, the forming of small clots, much smaller than those which are formed when this test is used for sour milk. Later on we got similar results with milk from cows suffering from lung tuberculosis from an abscess on the jaw and from inflammation of the kidneys. In a case of pyelonephritis the acidity was too high. The number of leucocytes was usually normal, sometimes a little greater.

In contrast to the milk of udder-diseased cows there was no great difference between the milk of the four quarters. Our experiment with turpentine injection, in which the temperature of the animal rose only slightly, proves that an aseptic inflammation in any other part of the body had only very little influence on the milk. It may be that toxic substances formed by bacteria can cause the secretion of abnormal milk.

Table II gives some of the results, which gave us in 1921 the impression that the importance of streptococcus is not so great as is usually believed. Several of the samples were highly abnormal both in appearance and composition. We usually analyzed the milk of each quarter separately. In several cases the milk of the other quarters was examined at the same time; not all these results are mentioned in the table. Table II gives the data of the milks without streptococci. No. 112 contained only a small number, Nos. 164 and 142 were rich in streptococci.

TABLE II—*Abnormal Milks, All without St*

No.	Acid-ity.	Oxa-late acid-ity.	pH	Sediment.	Leucocytes.	Non-protein nitro-gen.	C i
						mg. per 100 cc.	■ 1
92	2.6			1.4 per cent.		34.0	
94	5.2					40.5	
96	5.6	1.3		1.3 per cent.		34.5	
	6.4	1.8		2.1 " "		34.5	
97	3.36	−0.8		± 3 " "		52.3	
98	4.8	−0.2	6.82–6.9	0.8 " "		55.4	
110	6.0	1.2	6.75	0.2 " "	A great number.	46.6	
112	6.02	1.6	6.67–6.75	0.9 " "	" " " A few streptococci.	37.0	
131	6.2	3.7		3.5 " " A little blood.		68.6	
154	6.4	±0		±2.5 cc.	Full of leucocytes.	60.6	
161	6.8	2.6		0.6 per cent.		38.1	
162	7.8	4.8		± 3 cc.	Full of leucocytes.	60.2	
167	±4.0	±0.6		± 3 "	A great number.	46.7	
170 and 171	3.2	0.2	6.98	2.3 per cent.	" " "	40.0	
180	2.8	0.0	± 6.9–6.98	A little blood. ± 1.7 per cent.	" " "	39.2	
206	5.4	2.2		±5 per cent.	Full of leucocytes.	133.3	
207	4.0	0.8		±1.5 cc. A little blood.		63.4	
208	5.0	−1.4		±4 per cent. A little blood.	Full of leucocytes.	78.4	
230	5.1	0.85	6.75–6.82	0.4 per cent.	Large number.		
230a	5.1	0.85		0.4 " "		27.6	

xcept Nos. 112, 142, and 164.

Fat.	Casein.	CaO	Trypto-phane in serum.	Quarter.	Remarks.
per cent	per cent	mg. per 100 cc.	mg. per 100 cc.		
		206.4			Milk from 4 quarters (Cow 456); appeared nearly normal; a few small clots.
				L.F.	From Cow 456. The milk of the L.H.
				L.F.	and R.H. quarters contained strep-
				R.F.	tococci.
		88.0		L.F.	
2.6		252.0	High.		
2.25	2.47	214.0		L.H.	14 days after calving.
2.55	1.18	198.7	Normal.		14 " " " Injected with streptococcic serum before the examination of milk.
2.7	1.085		High.		13 days after calving. Milk from 4 quarters. 12 days later streptococci present.
		55.6		R.H.	Appeared very abnormal. Other quarters (i.e. L.F.) gave normal milk.
4.0		166.3		R.H.	14 days after calving. Appeared very abnormal.
2.1	3.1	99.3	Very high.	L.H.	5 days after calving. Appeared very abnormal.
1.048		130.8			4 quarters, at the end of lactation appeared very abnormal.
			High.	R.H. and R.F.	From Cow 456, some days earlier streptococci were present.
			"		4 quarters, 14 days after calving, appeared abnormal.
4.8		189.7		R.F.	In full lactation, appeared very abnormal, lesion of teat; one of the quarters (L.H.) gave milk with some streptococci.
				L.F.	Same cow as No. 206.
6.2		263.8		R.H.	" " " " 206. (catalase 5.2).
		137.7	Normal.	R.F.	
		137.7	14.5	R.F.	In full lactation, no diseases to be found.

TA[

No.	Acid-ity.	Oxa-late acid-ity.	pH	Sediment.	Leucocytes.	Non-protein nitro-gen.
						mg. per *100 cc.*
230b	6.24	1.73		0.4 per cent.		30.0
230c	6.2	1.43	6.65–6.72	0.5 " "		
230d	5.73	1.33	6.6 –6.65	0.4 " "		34.3
255	2.4				± 2,480,000 leucocytes per cc.	
256	3.6		±6.98		±1,000,000 leucocytes per cc.	
257	5.4		6.82–6.9		±280,000 leucocytes per cc.	
261	2.0	−2.05				
260	2.0	−1.95				
263	2.0	−2.0		± 3.0 cc.	±20,000,000	
262	3.8	−1.8		±1.5 "	± 2,320,000	
265	1.2	−1.68	6.9 –6.98		± 3,240,000 leuco-	45.4
264	2.16	−0.88			± 1,200,000 cytes.	41.0
267	4.61	±0.1	6.75–6.82		± 450,000	
266	5.93	±0.27	6.67–6.75		± 160,000	
297	±6.4					73.7
					Milk samp	
164	4.8	1.2	6.8 –6.9	± 1.5 cc.		
142	5.6	0.8	6.75–6.82	0.5 per cent		

cluded.

	Fat.	Casein.	CaO	Trypto-phane in serum.	Quarter.	Remarks.
	per cent	*per cent*	*mg. per 100 cc.*	*mg. per 100 cc.*		
2			179.0	16.6	L.F.	Same cow as No. 230a; the milk yields of this cow were abnormally small.
					R.H.	Same cow as No. 230a.
			186.0	16.3	L.H.	" " " " 230a.
					R.H.	Cow with inflammation of the throat and of the udder; however, no clinical mastitis could be detected by the veterinarians.
					L.H.	Same cow as No. 255; appeared very abnormal, like No. 255.
					L.F.	
					R.H.	3 days later than No. 255, appeared very abnormal. The clots make the determination of the leucocytes unreliable.
					L.F.	Same cow as No. 255.
7	Very little.		119.0		R.H.	}2 days later than No. 261, appeared very abnormal.
8			210.8		L.F.	}Same cow as No. 255, No. 262 without reductase.
			173.5		R.H.	}2 days later than No. 263; same cow as No. 255.
					L.F.	}Appeared less abnormal, catalase of both = 7.
			274.4		R.H.	}2 days later than No. 265; same cow as No. 255.
			203.0		L.F.	}Reductase test discolored within 2 hours.
			298.0			
	ng streptococci.					
			101.0	3×normal	R.H.	10 days after calving.
			188.0		L.H.	

The table mentions whether or not the milk was produced soon after calving. From the remarks made in the last column it may be seen that not infrequently at the same time that abnormal milk without streptococci was drawn from one quarter, other quarters gave milk with some streptococci. We also found that a quarter giving milk without streptococci 1 day produced at another time milk containing these bacteria in great numbers.[4] One of the striking features was that *in milk with a low acidity (less than about 3) streptococci were never present.* Perhaps one might conclude therefrom, that when the milk is highly abnormal it is no adequate medium for these bacteria. Abnormal milks, with or without streptococci, show acidities low (4 to 6) or normal (7 to 8). This was found in milks analyzed when quite fresh (*i.e.* within half an hour). The acidity is therefore no good criterion for streptococcic invasions in the udder. The high acidity sometimes found in streptococci-containing milk may in some cases be explained by the character of colostrum, the milk still possessed as colostrum has a high acidity; *i.e.*, of 17. In other cases it may be caused by the acids formed by the streptococci.

We found in sterilized milk, by infecting with *Streptococcus mastitidis bovis* and keeping at 37°C. for 3 days, the acidity increased from 8 to 24.4 and a second time from 8.4 to 29 in 2 days. Infection of sterilized milk with *pyogenes* had no or only a negligible influence on the acidity. We wish to point to the result of the generally used reduction test with methylene blue obtained on Sample 262. There was no reduction; *i.e.*, no discoloration, even in 24 hours.

In our abnormal samples without streptococci, as well as in those containing these bacteria, we found the *chlorides and tryptophane greatly increased, the lactose decreased.* As udder diseases often occur shortly after calving, it is necessary to take into account that the chemical criteria for abnormal milk give values for colostrum that resemble more or less those for milk of diseased udders.

[4] A more detailed report of our work is published in *Tydschr. Vergelijkende Geneesk.* (*J. Comparative Med.*), 1922, vii, official organ of the Society for the Knowledge of Milk.

The *non-protein nitrogen* gave with normal milks the same values when the proteins were precipitated with tungstic acid (Folin) or when metaphosphoric acid (25 per cent) was used for this purpose. This does not hold good for colostrum nor for abnormal milks. We will try to find the reason for this difference.[5] The non-protein nitrogen of abnormal milks is frequently higher than of normal milk, *i.e.* about 60 mg. instead of about 30 to 40 mg., as well as when streptococci are present in the absence of these bacteria. Nevertheless, the determination of this value is no sharp method for the detection of abnormal milk. In the above mentioned sample of milk, which was infected with streptococci we found no increase of the non-protein nitrogen. On the contrary, infecting sterilized milk with *pyogenes* raised the figure from 30 to 200 mg.; ammonia alone was almost formed. From the cow injected with silver nitrate solution only once did we determine the non-protein nitrogen; it was 53.3 mg. in 100 cc. The amount of non-protein nitrogen which we found in several samples of colostrum was always higher than in normal milk, usually 60 to 120 mg. in 100 cc.

In venous blood from the jugular vein of cows we found figures between 23 and 36 mg., shortly after parturition. That these figures are much lower than those of colostrum may be connected with the much higher content of nitrogenous substances in colostrum than in blood.

Concerning our *tryptophane* results obtained in 1921 and with the milk of the cow which was injected with silver nitrate, the following may be mentioned. Normal serum of milk, after precipitating the casein and the fat with a saturated solution of potassium-aluminium sulfate, contains about 14 to 20 mg. of tryptophane in 100 cc. In abnormal milks the amount is usually ten to fifteen times as high. *Of colostrum* prepared in the same way the tryptophane content is usually more than 0.5 gm. in 100 cc. The amounts of tryptophane, we found in abnormal milks, according to our results, approach that for blood serum. Milk contains no free tryptophane either in the ultrafiltrate or in the filtrate obtained after precipitating the proteins. Tryp-

[5] Some years ago in this laboratory differences were found in the non-protein nitrogen of blood by precipitating the proteins with different acids (Sjollema, B., and Hetterschy, C. W. G., *Biochem. Z.*, 1917, lxxxiv, 371).

TABLE III.
Samples of Milk from Udders That Were Examined Microscopically.

No.	Acidity.	Oxalate acidity.	Sediment.	Leucocytes.	CaO *mg. per 100 cc.*	Non-protein nitrogen *mg. per 100 cc.*	Remarks.
359	10.9, after 24 hours.	6.28, after 24 hours.	6 per cent with blood.	Too many to count.	272		Interstitial tissue of the udder greatly developed. Nearly all acini filled up with a homogeneous substance, which was evenly stained red by eosin. No symptoms of inflammation, no bacteria.
347a R.F.	6.2		0.25 per cent.	More than normal.			Tissue, R.F. and R.H., almost normal. The lumina of the alveoli are wide. The tissue of the L.F. has many alveoli with pus, containing polymorphonuclear leucocytes. The nuclei strongly stained with hematum.
347b R.H.	6.0		0.3 percent.				
347c L.F.	2.9		0.5 cc.	Too many to count.	208		
302	2.4			Appearance nearly normal, a fair amount of epithelial cells, a small number of leucocytes.	295	40	No microorganisms. Chronic interstitial mastitis. Most glandular alveoli without content some with a substance that is stained red with eosin. In the alveolar lumina a very small number of cells, no polymorphonuclear nor polymorphonu- clear leucocytes. The inter-

304	3.3	1.65	Coffee - colored, odor of putrefactive processes, neither streptococci nor leucocytes.	103	68	Normal udder in involution. No bacteria.

tophane could be detected. The very high tryptophane figures for colostrum can probably be explained by the high percentage of globulins. We determined a few times the amount of tryptophane in normal milks and in blood plasma (of cows) without treating them with potassium-aluminium sulfate or any other protein-precipitating reagent. We found about 100 mg. in 100 cc. of milk and about 400 to 600 mg. in blood plasma.

Our experience is not sufficient to state that at the end of the lactation period tryptophane increases in the milk. We are examining this point as well as others connected with the tryptophane question; *i.e.*, in how far the identity of the proteins of blood plasma and lymph with those of abnormal milks can be examined by means of the estimation of tryptophane and whether the amount of tryptophane is increased in abnormal milks if the affection that influences the milk secretion is somewhere else than in the udder.

In reference to the last point we refer to the data of Milk 230. The samples of the different quarters gave normal tryptophane figures. There was no udder disease to be found nor any other disease; the milk yields were very low. They were abnormal with regard to several points: acidity, chlorides, lactose, leucocytes, and catalase. It may be considered to be of importance, that we found leucocytes to be poor in tryptophane; therefore a direct relation between the number of leucocytes and the amount of tryptophane does not exist. We wished to know whether the streptococci were retained by the udder tissues in those cases in which milk of abnormal chemical composition contained no streptococci. If so, the conclusion from the microscopic examination of the milk would have been incorrect. For this purpose we analyzed the milk of animals brought to the abattoir to be slaughtered owing to serious udder diseases.

Mr. H. S. Frenkel kindly examined parts of the udder tissues microscopically to find out whether streptococci were present and to determine the nature of the mastitis. We cannot guarantee that the cows were in full lactation and regularly milked. As seen from the data given in Table III we found that samples of milk of a very abnormal composition were at the same time devoid of streptococci. It is, of course, impossible to state, that in these cases streptococci originally played no rôle. Two objec-

tions may be made. The first is that we made no cultures; *i.e.*, that we made no second microscopic examination after incubating the milk for some time at 37°C. We do not think it probable, that when streptococci cannot be detected, either in the milk or in the udder tissue, that they actually have a great influence on the milk secretion. The second objection may be that milk drawn at irregular times is not normal milk. Yet it is evident, that not all the abnormalities which the samples show, can be caused in that way; *i.e.*, Milk 347, a, b, and c drawn from different quarters differs greatly. An inflammation must have been the cause of this.

SUMMARY.

This paper shows that an aseptic inflammation of the udder, produced by an injection of a silver nitrate solution, causes the same changes of the milk as an infection with bacteria; *i.e.*, by streptococci. This renders uncertain the fact that streptococci usually originate the production of abnormal milk and of the inflammation of the udder.

An aseptic abscess produced in the region of the neck influenced the composition of the milk very little. The changes were not in the sense observed in udder diseases or in some other affections of the milch cow.

In some cases of serious udder diseases the udder tissues were devoid of streptococci. This holds good in cases in which the milk possessed the composition typical of streptococcic mastitis. Data are given of a number of samples of abnormal milk. The acidity of these samples was high, low, or normal. Independent of the acidity the amounts of chlorides, lactose, etc., were abnormal. Some other affections of the milch cow produce the same changes of the milk, except the increase of the number of leucocytes and the differences between the milk drawn from the four quarters.

Streptococci have little influence on the amount of non-protein nitrogen of the milk. *Pyogenes* increases this figure considerably.

The determination of tryptophane is one of the best reagents on milk of inflamed udders.

Colostrum serum contains much more tryptophane, for example 50 times as much, than normal milk serum.

An abnormally low acidity of milk can easily be detected with phenol red after adding potassium oxalate.

In milks with a very low acidity streptococci do not appear to be present.

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